Gene signatures with predictive and prognostic survival values in human osteosarcoma

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Osteosarcoma is a common malignancy seen mainly in children and adolescents. The disease is characterized by poor overall prognosis and lower survival due to a lack of predictive markers. Many gene signatures with diagnostic, prognostic, and predictive values were evaluated to achieve better clinical outcomes. Two public data series, GSE21257 and UCSC Xena, were used to identify the minimum number of robust genes needed for a predictive signature to guide prognosis of patients with osteosarcoma. The lasso regression algorithm was used to analyze sequencing data from TCGA-TARGET, and methods such as Cox regression analysis, risk factor scoring, receiving operating curve, KMplot prognosis analysis, and nomogram were used to characterize the prognostic predictive power of the identified genes. Their utility was assessed using the GEO osteosarcoma dataset. Finally, the functional enrichment analysis of the identified genes was performed. A total of twenty-gene signatures were found to have a good prognostic value for predicting patient survival. Gene ontology analysis showed that the key genes related to osteosarcoma were categorized as peptide–antigen binding, clathrin-coated endocytic vesicle membrane, peptide binding, and MHC class II protein complex. The osteosarcoma related genes in these modules were significantly enriched in the processes of antigen processing and presentation, phagocytosis, cell adhesion molecules, Staphylococcus aureus infection. Twenty gene signatures were identified related to osteosarcoma, which would be helpful for predicting prognosis of patients with OS. Further, these signatures can be used to determine the subtypes of osteosarcoma.

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Abstract

Osteosarcoma is a common malignancy seen mainly in children and adolescents. The disease is characterized by poor overall prognosis and lower survival due to a lack of predictive markers. Many gene signatures with diagnostic, prognostic, and predictive values were evaluated to achieve better clinical outcomes. Two public data series, GSE21257 and UCSC Xena, were used to identify the minimum number of robust genes needed for a predictive signature to guide prognosis of patients with osteosarcoma. The lasso regression algorithm was used to analyze sequencing data from TCGA-TARGET, and methods such as Cox regression analysis, risk factor scoring, receiving operating curve, KMplot prognosis analysis, and nomogram were used to characterize the prognostic predictive power of the identified genes. Their utility was assessed using the GEO osteosarcoma dataset. Finally, the functional enrichment analysis of the identified genes was performed. A total of twenty-gene signatures were found to have a good prognostic value for predicting patient survival. Gene ontology analysis showed that the key genes related to osteosarcoma were categorized as peptide–antigen binding, clathrin-coated endocytic vesicle membrane, peptide binding, and MHC class II protein complex. The osteosarcoma related genes in these modules were significantly enriched in the processes of antigen processing and presentation, phagocytosis, cell adhesion molecules, Staphylococcus aureus infection. Twenty gene signatures were identified related to osteosarcoma, which would be helpful for predicting prognosis of patients with OS. Further, these signatures can be used to determine the subtypes of osteosarcoma.

1   Introduction

Osteosarcoma (OS) is a common primary malignancy seen mainly in children and adolescents(Yang et al., 2018). Its annual incidence ranges from 2 to 3 in 1 million people(de Azevedo et al., 2020), and it accounts for 40.51% of primary bone malignancies(Li et al., 2020). The incidence of OS is higher in males than in females(Damron et al., 2007), and it accounts for 15% of all extracranial tumors in the 10–19-year-old age group)(Nie & Peng, 2018). It is considered the third most common cancer in adolescence(Zhang et al., 2018). OS treatment is aggressive and combines neoadjuvant chemotherapy, extensive surgical resection, and additional postoperative adjuvant chemotherapy. Five-year survival rate of non-metastatic patients is 65%–70%(Siegel et al., 2018). Regarding patients with distant metastases, 5-year survival rate is only 15%–30%(Whelan & Davis, 2018). Further, relapse rate remains high at approximately 35%(Yu et al., 2014). Metastasis remains the main cause of death in patients with OS. Many prognostic factors are reported to be related to OS: miR-195, miR-21, TGF-β, MMP-9, HIF-1, APE1, and COX2. However, use of these factors has not improved survival rate related to OS(Zamborsky et al., 2019). Therefore, clarifying the molecular mechanisms underlying the occurrence and progression of OS and exploring the potential diagnostic and therapeutic targets are of great importance for the diagnosis and treatment of OS. Many biomarkers have been evaluated (Table 1)(Wan-Ibrahim et al., 2015), but none have been approved by the Food and Drug Administration for clinical settings (Wan-Ibrahim et al., 2015). This lack may be due to research deficiencies, unacceptable heterogeneity, or absence of effective evaluation. Biomarkers for patients with OS, particularly those with metastases, are urgently needed for early diagnosis and

71   establishment of treatment goals. Serum biomarkers are used for predicting prognosis of other
72   cancers but are rarely characterized in OS(Zamborsky et al., 2019). An obvious need in OS is
73   effective biomarkers for characterizing disease progression and associated prognosis.

74   One study reported that CDC20 and its downstream substrates, secure, cyclin A2 and cyclin B2
75   are good prognostic factors for OS(Wu et al., 2019). Savage et al(Jin et al., 2007). suggested that
76   two loci in the GRM4 gene at 6p21.3 and in the gene, desert, at 2p25.2, These two loci warrant
77   further exploration to uncover the biological mechanisms underlying susceptibility to
78   osteosarcoma.The study addressed a single gene and did not take into account interactions
79   among molecules that regulate tumorigenesis. Three candidate genes (ALOX5AP, CD74 and
80   FCGR2A) were found. Their expression levels in lung and lymph nodes were higher than levels
81   in matched cancer tissues, and they may be expressed in microenvironments(Li et al., 2020).
82   Some limitations exist in these studies. First, accuracy cannot be guaranteed with only one
83   dataset because of an expected high false-positive rate. Further, using a single high-throughput
84   analysis method (only sequencing or chip data), results obtained will be biased. Second, a
85   patient's sample data are too limited. Finally, clinical information is incomplete.

86   Identifying the minimum number of robust genes needed to produce a predictive signature for
87   prognosis for patients with OS was the objective of this study. The lasso regression algorithm
88   was used to analyze sequencing data from TCGA-TARGET, and Cox regression analysis, risk
89   factor score, receiving operating curve (ROC), KMplot prognosis analysis, nomogram and other
90   methods were used to assess genes for their predictive power. Next, the accuracy and predictive
91   power of twenty-gene identified in this process were assessed using the GEO OS dataset. Finally,
92   we performed functional enrichment analysis on these twenty-gene.

93   **2   Material and Methods**

94   **2.1   Data collection and preprocessing**

95   Training set: The TARGET-OS RNA-sequencing dataset (presented as fragments per kilobase
96   million, FPKM), corresponding clinical characteristics and prognosis information were
97   downloaded from UCSC Xena (Goldman et al., 2019)(https://xena.ucsc.edu/). Patients with
98   expression profiles but no prognostic information and clinical characteristics were excluded.
99   Finally, 84 patients with OS were included in a training set. FPKM data were converted to TPM
100 data and annotated using gencode.v22.annotation.gene.probeMap.

101 Validation set: The gene expression data GSE21257(Buddingh et al., 2011) (GPL570 (HG-
102 U133_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array) for 53 patients with OS were
103 downloaded from the GEO database, and corrected and annotated with R software.

104   **2.2   Construction of gene signatures**

105 A linear regression multiple regression model was developed for the underlying expression
106 levels of genes for prognostic risk scores. The method chosen by lasso Cox was 10-fold cross-
107 validation. According to the median cutoff value (the cutoff value refers to the content before the
108 brackets of the HR value in each dataset) of the risk score, patients with OS were divided into
109 high-risk and low-risk groups. Model prediction efficiency using the training set was evaluated
110 by Kaplan–Meier log-rank test, time-dependent ROC curve analysis, Cox regression analysis,

111   and risk factor score for validation and test sets. A nomogram was constructed using Iasso's
112   guidelines.

2.3   Weighted correlation network analysis of genes

114   Based on the variance of gene expression in TARGET-OS data, the top 5000 genes were selected
115   for WGCNA(Langfelder & Horvath, 2008). This analysis proceeded as: check for outliers in all
116   samples, construct sample tree with hclust, and remove outliers according to cut height. To
117   explore the correlation between expression data and clinical phenotypes, the sample tree and
118   characteristic heat map were visualized. Subsequently, the strength of associations between pairs
119   of nodes of the adjacency matrix aij was calculated as: sij = | cor(xi,xj)| aij =Sijβ. xi and xj
120   are genes i and j. The vector of expression value, sij, indicates the strength of Pearson's
121   correlation coefficient between genes i and j. The aij coding network connects genes i and j. β
122   value is a soft threshold (power value). Further, the Scale-Free Topology Fit Index (scale-free
123   R2) range from 0 to 1 is used to determine the scale-free topological model. Selecting a set of
124   soft threshold powers (range: 1 to 20) assists in calculating scale-free topological model fitting.
125   The soft threshold of β=7 was used to define the adjacency matrix. The corresponding scale-free
126   R2 value is 0.87, suggesting a satisfactory scale-free topology model.

127   In coexpression networks, the highest absolute association genes were clustered into the same
128   module to generate a clustering dendrogram. Relationships between clinical traits and risk scores
129   were analyzed by Pearson correlation and results were visualized by heat map analysis. Genes in
130   the module were analyzed for gene ontology (GO) and KEGG pathway enrichment. Moreover,
131   Cytoscape(Smoot et al., 2010) (version 3.7.2) was used to visualize the weighted coexpression
132   network. gene ontology (GO) analysis, which includes annotation of biological processes (BPs),
133   molecular functions (MFs), and cellular components (CCs), serves as a major bioinformatics tool
134   to annotate genes and analyze the biological processes of these genes. Huimei Wang et al
135   analyzed the biological classification of DEGs, showing that changes in BPs of DEGs were
136   significantly enriched in positive regulation of associated cell response, by GO analysis(Wang et
137   al. 2018).

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2.4   Functional enrichment analysis

140   Clusterprofiler(Yu et al., 2012) was used to study modules related to biological function for
141   determining functional and pathway enrichment. A multiple testing correction was performed
142   using hypergeometric test functions and the Benjamini–Hochberg method. The GOplot (Walter
143   et al., 2015) package was used to visualize the enrichment analysis.

3   Statistical analysis

145   Statistical analysis was conducted in R software (version 3.6.1) with the following packages:
146   "glmnet"(Friedman et al., 2009), "survivalROC" (Heagerty et al., 2000), "WGCNA," and
147   "clusterProfiler." All the statistical tests were two sided, and P-values of <0.05 were considered
148   statistically significant.

149 **4   Results**

150   **4.1   Construction of genes classifier for OS**

151   Prognostically significant genes for OS in TARGET-OS data were analyzed and a total of 1151
152   genes were significantly associated with poor prognosis. These 1151 candidate genes were
153   included in the lasso prognostic classifier for further screening and model construction. A
154   twenty-gene classifier for the OS was developed (Figure 1A and 1B). The gene information in
155   the model is shown in Table 1. Patients were divided into a high-risk group (n = 42) and low-risk
156   group (n = 42) based on risk scores. The median risk score was set as the cutoff (the cutoff value
157   refers to the content before the brackets of the HR value in each dataset). Table 2 shows clinical
158   characteristics of patients with OS in the training set according to their high risk and low risk
159   scores. The Kaplan–Meier log-rank test suggested a significant difference between high-risk and
160   low-risk groups in the training set ($P < 0.001$; Figure 1C). In the time-dependent analysis of the
161   ROC curve, AUCs for OS in the first, third, and fifth years were 0.94, 0.98, and 0.97,
162   respectively (Figure 1D).

163   **4.2   Identification of a prognostic risk score model based on the training set**

164   Based on univariate regression analysis (Cox's proportional hazard model), forest plots of
165   selected genes with *p-value* and hazard ratios are shown in Figure 2A. HR, Hazard Rate in
166   Figure 2A, shows that the p-value of selected genes was less than 0.05, showing the statistical
167   difference, which cannot be ignored. Only when 95% CI conclude 1, it could be proved that
168   these selected genes signatures were not significantly associated with the prognosis of patients
169   with OS. Although the extremum of HR is very close to 1, they show that these selected genes
170   might be associated with the prognosis of patients with OS. Risk scores and survival status of
171   patients with OS are shown in Figure 2B and 2C, respectively. These results indicate that
172   patients with high risk scores have poor outcomes compared with patients with low risk scores
173   (Figure 2D). Using these data, a nomogram combining the classifier with clinicopathological
174   features to predict the survival probability of patients with different risk scores was prepared
175   (Figure 3A). The calibration chart demonstrated that predicted three-year and five-year survival
176   rates were very close to observed ratios (Figure 3B).

177   **4.3   Validation of twenty-gene signature for survival prediction in the validation set**

178   Subsequently, the validation set was used to assess the power of the twenty-gene signature in
179   predicting prognosis. OS of the low-risk group was superior to the high-risk group ($P < 0.05$;
180   Figure 4A). Also, time-dependent ROC analysis is used to assess the effectiveness of risk models
181   in predicting outcomes. Areas under ROC was 0.81, 0.78, and 0.76 at one, three and five years,
182   respectively, suggesting that the twenty-gene signature displays good predictive power (Figure
183   4B). The C-index value is 0.944. The risk score (Figure 4C) and survival status (Figure 4D) of
184   patients with OS, and distribution of risk scores of twenty-gene expression profiles are shown in
185   a heat map of 53 patients in the validation set (Figure 4E). These results suggest that the twenty-
186   gene signature shows good prognostic value for patient survival. This finding is further validated
187   since patients with high risk scores were associated with poorer prognoses compared with
188   patients with low risk scores. Previous studies have investigated gene factors in an attempt to
189   identify new prognostic OS markers**(Goh et al. 2019; Guan et al. 2020)**. Hence, I have

190 compared the prognostic gene set for osteosarcoma, Cox univariate and multivariate analysis
191 showed that BACE2, ING2 ALOX5AP, HLA-DMB, HLA-DRA, and SPINT2 were not the
192 independent prognostic factors for osteosarcoma (Table 3). Table 3 shows the determinants of
193 BACE2, ING2 ALOX5AP, HLA-DMB, HLA-DRA, and SPINT2 for all the patients. In the
194 univariate analysis, These gene signatures exhibited a negative correlation with age, gender,
195 specific and primary tumor site, but illustrate a positive correlation with recurrence and risk
196 score. After multiple stepwise analysis, these biomarkers correlated independently with
197 Recurrence (hazard ratio (HR) 5.374, 95% CI 1.183-24.404, p =0.029) and risk score (HR 9.869,
198 95% CI 4.663-20.887, p < 0.001).

199

4.4 Gene co-expression network analysis

201 The relationship between risk scores and gene expression profiles was evaluated using the top
202 5000 genes in the variance filter for WGCNA analysis. The red line (cut height = 8000) was used
203 to remove the abnormal samples in the sample tree. TARGET-40-PAUXOZ-01A was excluded
204 after removing outliers (Figure 5). The sample dendrogram and trait heat map placed selected
205 samples into different sample clusters that provided clinical trait information (Figure 6A).
206 Independence and average connectivity of coexpression modules were determined by power (β)
207 and scale R^2 value. A series of soft thresholds and corresponding performance power were
208 plotted. The threshold for scale R^2 value was set at 0.85. The power value of seven is the
209 threshold that first reaches the scale R^2 value, and was chosen as the soft threshold to construct
210 and identify coexpression modules (Figure 6B and 6C). The expression matrix was converted to
211 an adjacency matrix, and subsequently into a topology matrix. Based on TOM, genes are
212 clustered based on criteria of mixed dynamic shear trees using average connection–level
213 distance. The minimum number of genes in each module was set at 7. Finally, a total of 11
214 modules were identified with the WGCNA package (Figure 6D). Statistics of gene numbers in
215 each module are shown in Table 4. Associations of these modules with clinical characteristics
216 (including sex, age, relapse, OS, metastasis, major cancer sites, specific cancer sites, and risk
217 scores) are shown in Figure 6E. T module showed the highest correlation with the risk score.
218 Further, the magenta module also was negatively correlated with sex and OS and positively
219 correlated with major cancer sites. GSEA analysis between high-risk and low-risk groups can
220 better illustrate the risk-score related to the biological process. But GSEA analysis between high-
221 risk and low-risk groups only considers the expression level of gene sets. Analysis of WGCNA is
222 based on the clinical relevant factors, which makes it sense, although its risk-score is low.

4.5 Functional enrichment analysis in the gene coexpression network of the magenta module

225 GO and KEGG enrichment analysis was used to characterize biological functions of genes in the
226 magenta module as they related to risk scores. GO analysis showed that key genes related to OS
227 were mainly enriched in peptide–antigen binding, clathrin-coated endocytic vesicle membrane,
228 peptide binding, and MHC class II protein complex (Figure 7A). KEGG analysis showed that the
229 key genes related to OS were mainly enriched in antigen processing and presentation,
230 phagosome, cell adhesion molecules, *Staphylococcus aureus* infection (Figure 7B).
231 Subsequently, the interaction network for enrichment pathways in the magenta module was

232   visualized (Figure 7C). Moreover, an interaction network was constructed to visualize genes in
233   the coexpression magenta module (Figure 7D).

234   **5   Discussion**

235   OS is a disease involving complex interactions among many factors. Overall, OS disrupts cell
236   signaling pathways, causing loss of bone tissue homeostasis(Otoukesh et al., 2018). The urgent
237   need to obtain better clinical results highlights the related need for better diagnostic, prognostic,
238   and predictive biomarkers(Ludwig & Weinstein, 2005). Presently, no specific markers are
239   available for OS diagnosis. To reduce mortality and increase limb salvage, biomarkers are
240   needed for early identification of disease(Smida et al., 2017). Possible genetic biomarkers to
241   address this need were identified in the present study. Lasso regression screened in twenty-gene
242   in a training set. These genes have certain prognostic value in time ROC, risk factor, and
243   Kaplan–Meier plots. Results were verified with a validation set.

244   One current study showed that inhibition of BNIP3 expression by baicalein treatment could
245   inhibit cell apoptosis(Ye et al., 2015). Moreover, the MYC gene is also reported to be amplified
246   in a subset of OS(Ladanyi et al., 1993). MYC promotes the proliferation of OS cells through the
247   autophagy pathway(Mo et al., 2019). Cross-species genomics identified DLG2 as a tumor
248   suppressor in OS(Shao et al., 2019). Further, depletion of KIF25 leads to the formation of actin
249   stress fibers, which may be due to the changes of Rho signaling observed before microtubule
250   destabilization(Wittmann & Waterman-Storer, 2001). In addition to the above studies of genes
251   related to OS, relationships between other genes screened in this study and malignancies have
252   been reported in the literature but have not been reported in OS. A lack of related research
253   reports to help assess the potential of these genes as targets for the treatment and diagnosis of OS
254   currently exits.

255   The current study also explored the relationship between risk scores and gene expression profiles
256   using WGCNA analysis. Important genes related to OS were enriched in 11 different modules.
257   Results showed that pathways related to inflammation and immunity were primarily enriched in
258   the turquoise module. The gray and turquoise modules share the most pathways among all
259   pairwise comparisons. Genes in these modules may play similar roles in OS.

260   LASSO is a method of shrinking and variable selection linear regression model. The purpose of
261   LASSO regression is to obtain a subset of the predictors to minimize the prediction errors of the
262   quantitative response variables. The lasso does this by constraining the model parameters,
263   making the regression coefficients of some variables approach 0. "Coxnet" fits a lasso penalty,
264   and its adaptive forms, such as adaptive lasso. Moreover, it treats the number of non-zero
265   coefficients as another tuning parameter and simultaneously selects with the regularization
266   parameter "lambda". And Yanlu Xiong et al established a 13 gene-based survival score for
267   prognostic prediction of Lung adenocarcinoma.They filtrated the relevant gene by LASSO and
268   identified the wide applicability of these genes (Xiong et al., 2020).

269   Previous studies report that CXCR3 may be an independent prognostic risk factor, suggesting a
270   possible benefit of immunotherapy for OS(Tang et al., 2019). The susceptibility and severity of
271   OS may also be related to functional polymorphism of inflammatory genes(Oliveira et al., 2007).
272   Another study has shown that increased expression of MIF indicates an increased risk of
273   metastasis, and MIF is related to angiogenesis and cell infiltration of OS. MIF can be used as a

274   prognostic marker of OS and a potential therapeutic target(Kim et al., 2008). Therefore,
275   clarifying molecular mechanisms of OS may facilitate the identification of novel therapeutic and
276   prognostic targets. Limitations to the study exist. First, although these twenty-gene were
277   identified to have certain prognostic value for OS, all data analyzed in the study were retrieved
278   from the online databases. Thus, further experimental evidence, such as real-time PCR, western
279   blot, immunohistochemistry assays, is required to fully elucidate the role of 20-gene signatures.
280   Second, to determine the diagnostic accuracy of gene associations, a larger sample size would be
281   useful for additional internal validation. Third, assessment of all clinically relevant influencing
282   factors for OS was not included, and more clinical information and PFS-related data are needed.
283   Finally, differentially expressed genes were not evaluated and nor was the need to add a normal
284   control group and joint verification of multiple tumor sites.In conclusion, the current study
285   proposes a 20-gene signature for diagnostic and prognostic purposes for OS. The twenty-gene
286   signature is independently related to prognostic parameters of OS classification. Also, the
287   signature is a good classifier for different subtypes of patients with OS. This signature may
288   provide a new perspective on the prognosis of OS. The biological functions and pathways
289   enriched in specific modules will be beneficial to the development of new therapeutic methods
290   for the treatment of OS.

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382

Figure 1

Construction of comprehensive prognostic classifier based on the training set.

A-10-fold cross-validation for tuning parameter selection in the LASSO model for OS.B-LASSO coefficient profiles of 20 prognostic genes for OS.C-Kaplan-Meier overall survival with a low or high risk of death in the training dataset.D-Time dependent ROC curves at 1, 3 and 5 years for OS☐ OS overall survival.

A

B

C

D

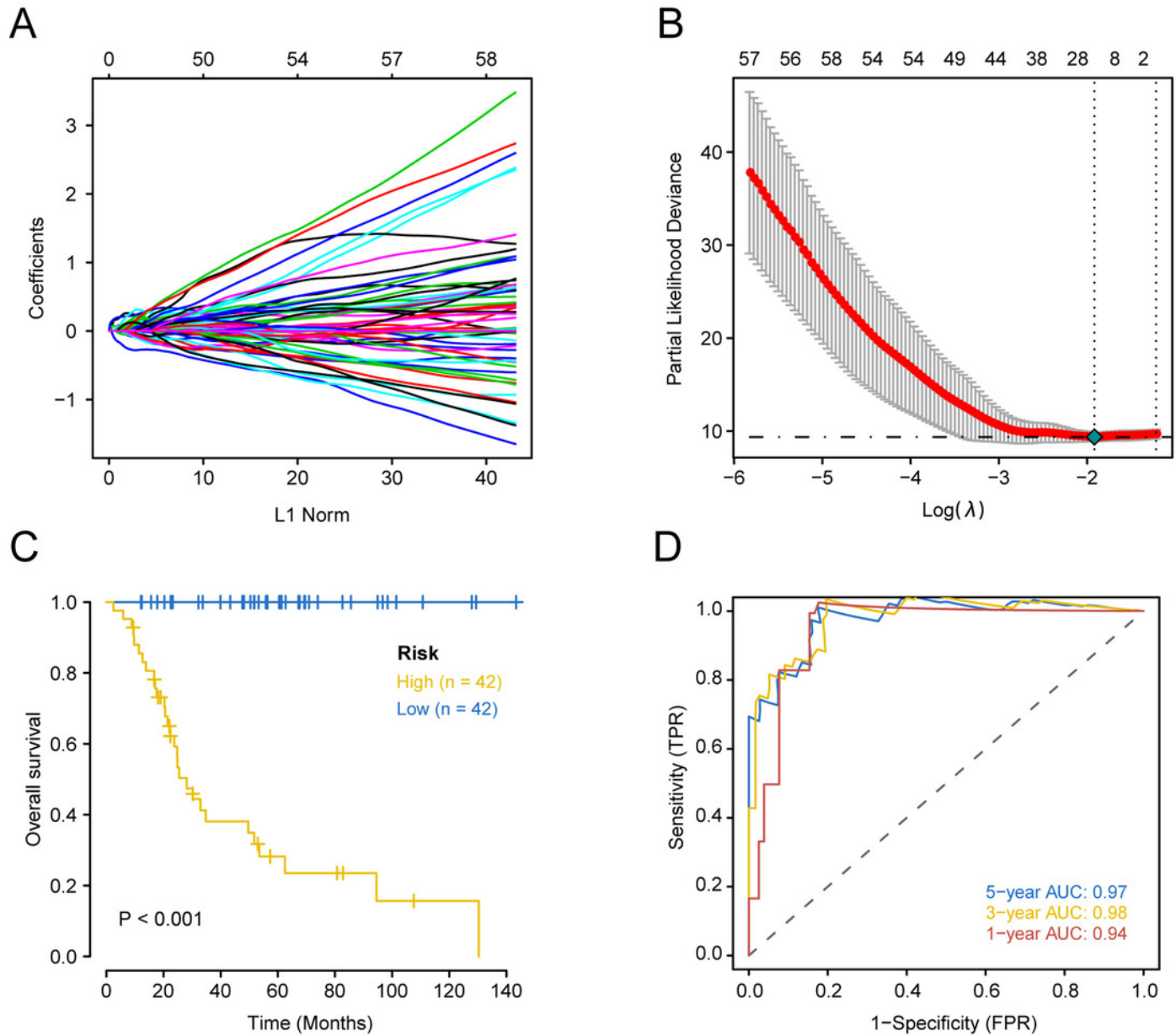



Figure 2

Multivariate Cox regression analysis was performed for the selected genes, and the risk scores, survival status and risk heat maps of the 20 prognostic genes were distributed in the training set

(A) Hazard ratio distribution for selected 20 key genes.distribution of risk scores, (B)overall survival of 84 patients, and heatmap of 20 genes in prognostic classifier in the training set. The black dotted line suggested the median cutoff divividing patients into low-risk and high-risk groups .

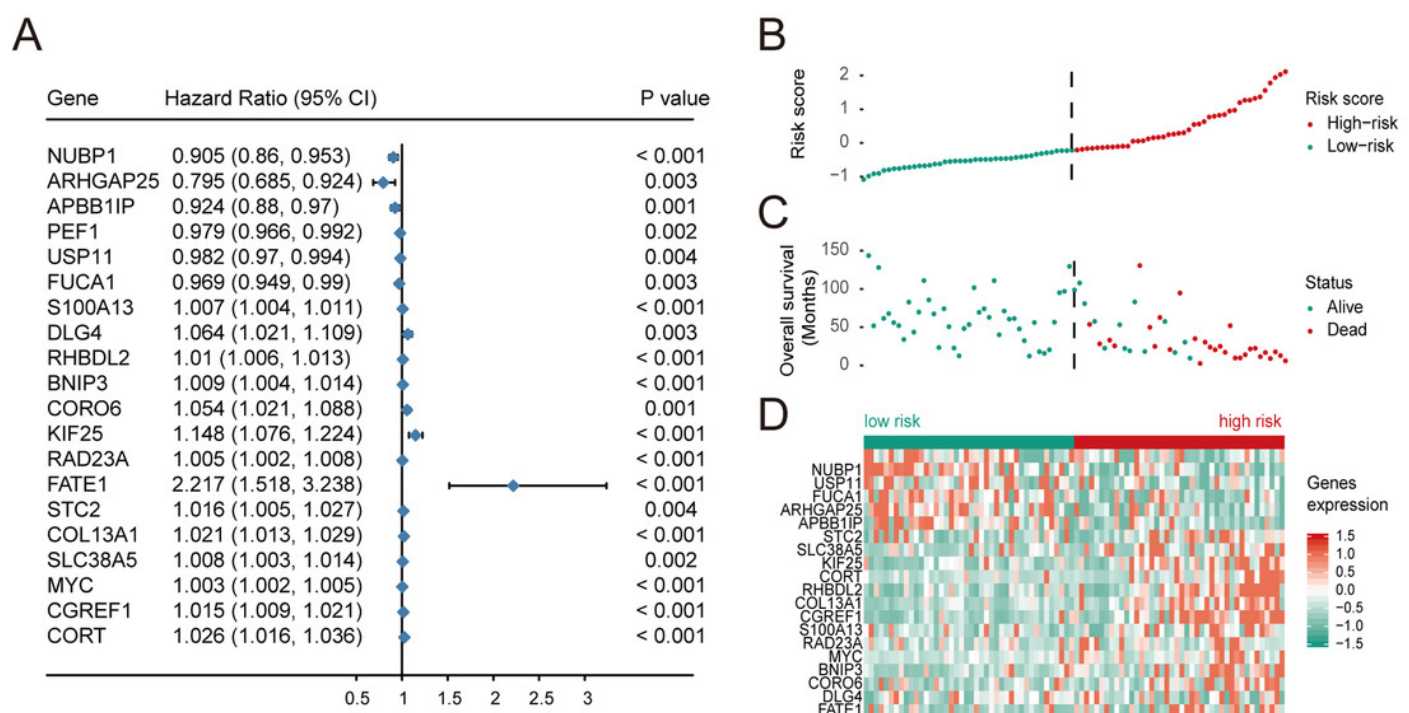



Figure 3

Nomograms to predict 1-year, 3 – year and 5-year survival probability in osteosarcoma

(A)Total points were obtained by incorporated the corresponding points of recurrence, metastasis, risk score on the point scale. The total points were then converted into specific 1 year, 3- yearand 5-year associated survival probabilities.(B)Calibration plot underlying the nomogram. Dashes show the nomogram-predicted probability for each group, as well as the respective confidence intervals.

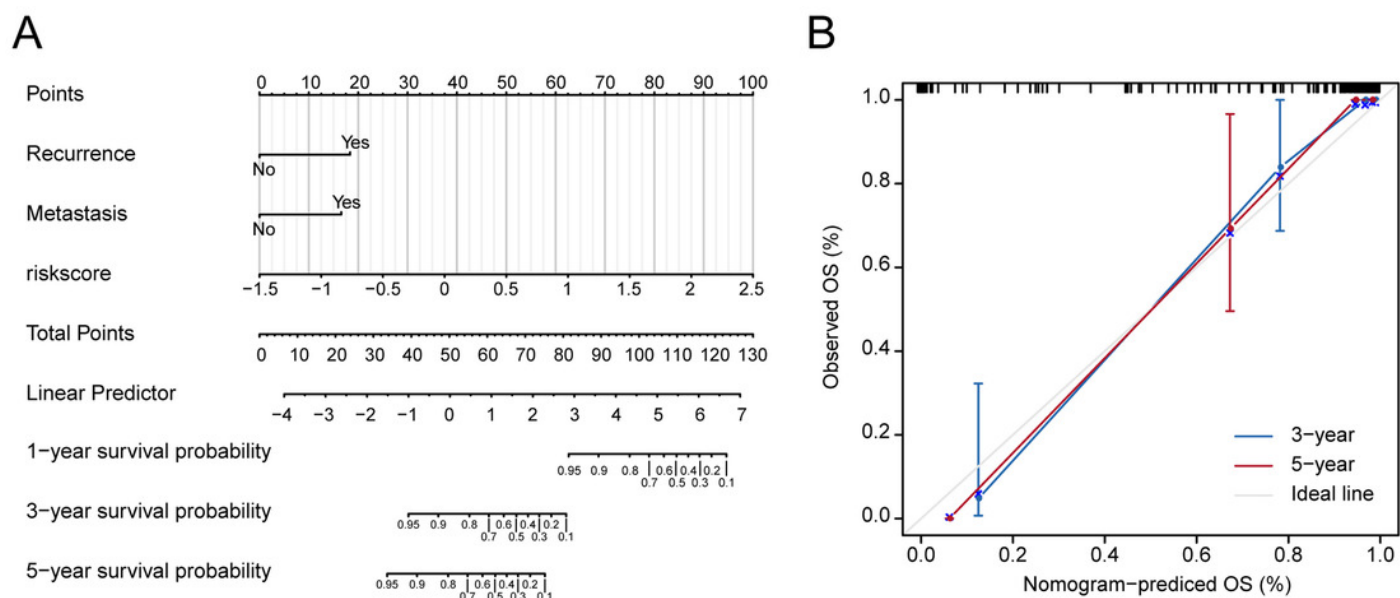



Figure 4

Kaplan-Meier survival, time-dependent ROC curves, and risk score distribution, the overall survival status of 84 patients, and heat maps of the expression of 20 genes in the low and high risk groups based on the validation set

(A)Kaplan-Meier curve of osteosarcoma patients with a low or high risk of death.(B)Time dependent ROC curves at 1, 3, and 5 years for OS in the validation set. (C)Distribution of risk scores for genes in the validation set ,(D) overall survival of 53 patients, (E) and heat maps of the expression of 20 genes in the low-risk and high-risk groups.

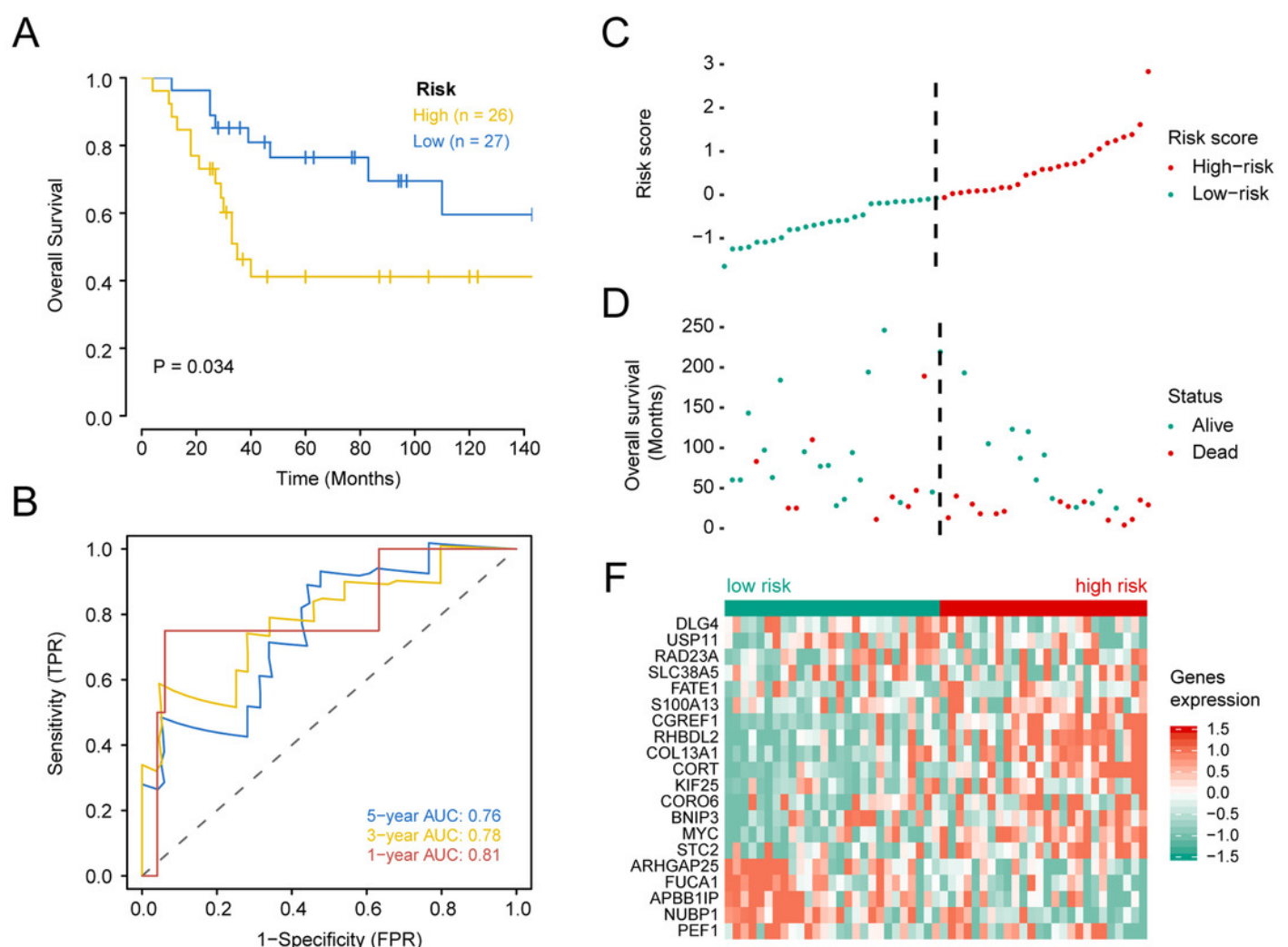



Figure 5

Sample cluster analysis to identify outliers underlying RNA sequencing data

The red line suggested the cut-off data filtering in the data preprocessing step c.

Figure 6

Overview of co-expression modules identified from the osteosarcoma RN

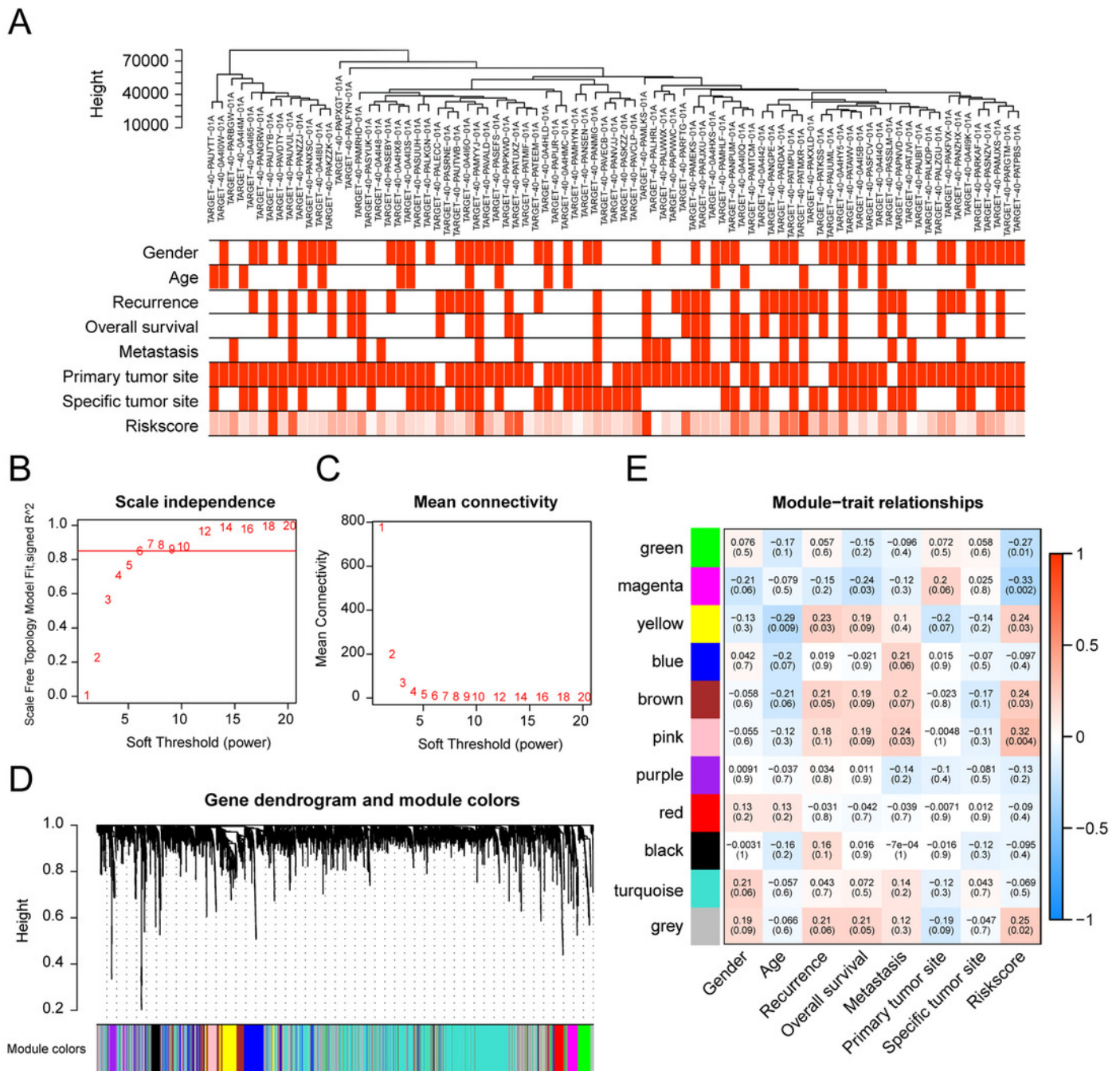
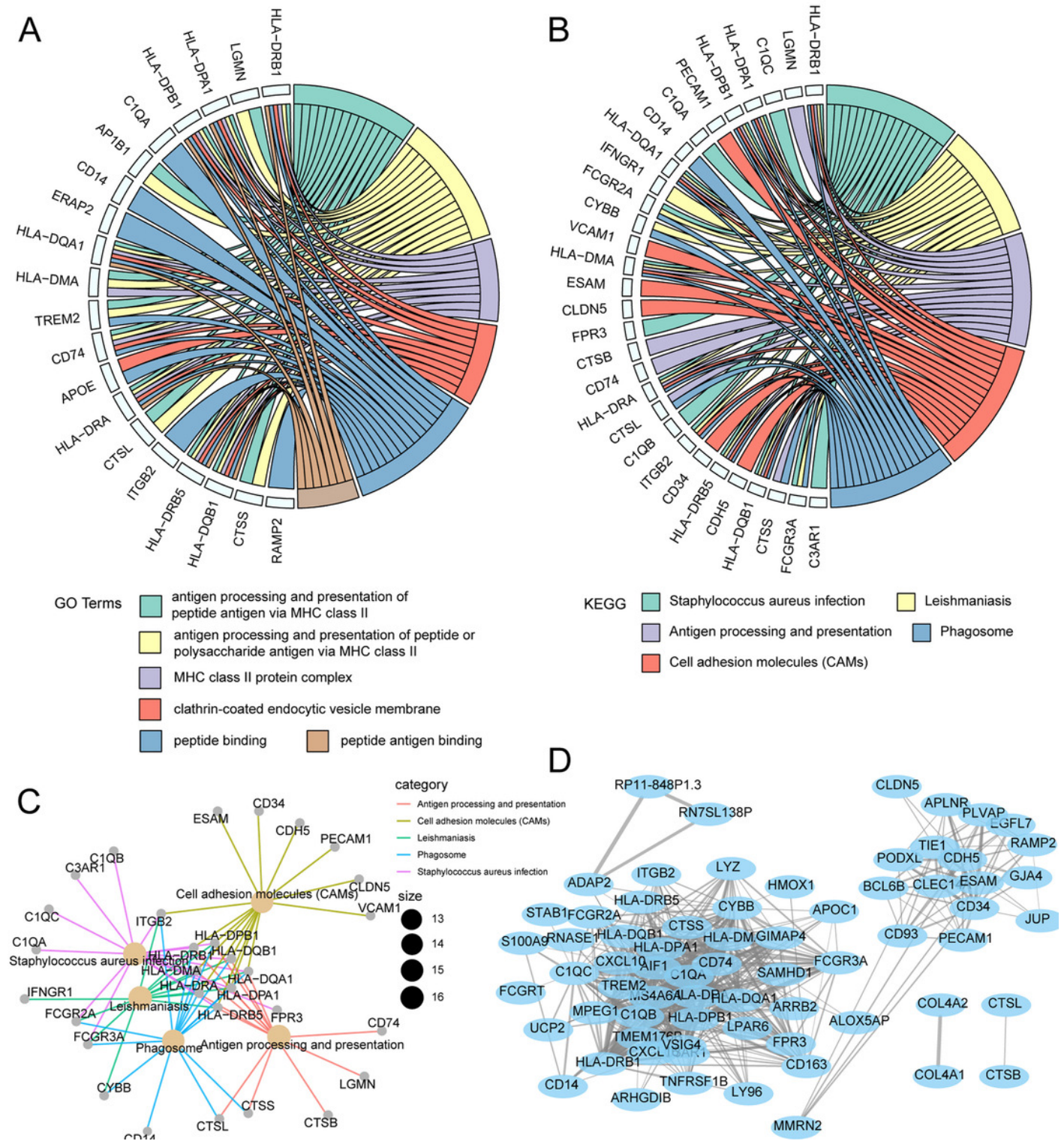



Figure 7

Functional enrichment analysis and WGCNA weighted network analysis of magenta module

(A) GOplot indicated the correlation between magenta module genes and their related GO terms. (B) GOplot indicated the correlation between magenta module genes and their related KEGG terms.(C) KEGG pathway analysis of magenta module genes based on clusterprofiler.(D) WGCNA weighted network diagram of magenta module.

Potential biomarkers for osteosarcoma

1   Table 1. Potential biomarkers for osteosarcoma

Study approach	Type of biomarker	Candidate biomarker	Regulation	Sample	Technique	Reference
Genomics	Diagnostic	C7orf24	↑	Tissue; cell lines	RT-PCR; qRT-PCR; Western blot (WB); siRNA transfection	(62)
	Diagnostic And Predictive	miRNA signatures	−	Tissue; cell lines	qRT-PCR	(58)
	Predictive	Multigene classifier	−	Tissue	cDNA microarray; qRT-PCR	(60)
	Predictive	Multigene classifier	−	Tissue	cDNA microarray; RT-PC	(61)
	Predictive And Prognosis	miRNA signatures	−	Tissue	miRNA microarray; qRT-PCR; immunohistochemistry (IHC)	(59)
	Predictive And Prognosis	miR-21	↑	Serum	qRT-PCR	(51)
	Prognosis	Tenascin-C	↓	Cell lines	cDNA microarray; RT-PCR; WB; IHC	(64)
	Prognostic	miRNA-214	↑	Tissue	qRT-PCR	(63)
Proteomics	Diagnostic	Serum amyloid A (SAA)	↑	Plasma	SELDI-TOF MS; WB	(71)
	Diagnostic	Ezrin (EZR); a crystallin β chain (CRYAB)	↑	Tissue	2D-DIGE; LC-ESI-MS/MS; RT-PCR for mRNA; tissue microarray and IHC	(66)
	Diagnostic	Cytochrome C1 (CYC-1)	↑	Serum; cell lines	SELDI-TOF MS; Gene microarray (cell lines)	(72)
	Diagnostic	Zinc finger protein 133 (ZNF 133); tubulin-a1c (TUBA1C)	↑	Tissue	2-DE; MALDI-TOF MS; WB; IHC	(70)
	Diagnostic	Protein NDRG 1	↑	Plasma membrane from cell line and tissue	2-DE; LC-ESI-MS/MS; IHC; WB	(67)
	Diagnostic	Gelsolin	↓	Serum	2D-DIGE; MALDIHTOF; WB; ELISA	(68)
	Predictive And Prognostic	SAA	↑	Serum	2D-DIGE; MALDI-TOF MS; WB; ELISA	(2)
	Predictive	Peroxiredoxin 2 (PRDX2)	↑(poor prognosis)	Tissue	2D-DIGE; LC-ESI-MS/MS; WB	(69)
	Predictive	SAA; transthyretin (TTR)	↑(poor prognosis)	Plasma	SELDI-TOF MS; WB	(73)

Twenty genes significantly related to the overall survival in the training set underlying the LASSO model

1   **Table 1** *Twenty genes significantly related to the overall survival in the training set underlying the LASSO model*

Ensemble ID	Gene	Chromosome location	Coefficient of lasso model	HR	pvalue
ENSG00000103274.9	NUBP1	chr16:10743786-10769351:(+)	-0.2216	0.905 (0.86, 0.953)	0.000
ENSG00000163219.10	ARHGAP25	chr2:68679601-68826833:(+)	-0.0503	0.795 (0.685, 0.924)	0.003
ENSG00000077420.14	APBB1IP	chr10:26438203-26567803:(+)	-0.0429	0.924 (0.88, 0.97)	0.001
ENSG00000162517.11	PEF1	chr1:31629862-31644896:(-)	-0.0230	0.979 (0.966, 0.992)	0.002
ENSG00000102226.8	USP11	chrX:47232690-47248328:(+)	-0.0123	0.982 (0.97, 0.994)	0.004
ENSG00000179163.11	FUCA1	chr1:23845077-23868294:(-)	-0.0104	0.969 (0.949, 0.99)	0.003
ENSG00000189171.12	S100A13	chr1:153618787-153634092:(-)	0.0019	1.007 (1.004, 1.011)	0.000
ENSG00000132535.17	DLG4	chr17:7189890-7219702:(-)	0.0088	1.064 (1.021, 1.109)	0.003
ENSG00000158315.9	RHBDL2	chr1:38885807-38941799:(-)	0.0144	1.01 (1.006, 1.013)	0.000
ENSG00000176171.10	BNIP3	chr10:131966455-131981931:(-)	0.0170	1.009 (1.004, 1.014)	0.000
ENSG00000167549.17	CORO6	chr17:29614756-29622907:(-)	0.0425	1.054 (1.021, 1.088)	0.001
ENSG00000125337.15	KIF25	chr6:167996241-168045089:(+)	0.0570	1.148 (1.076, 1.224)	0.000
ENSG00000179262.8	RAD23A	chr19:12945855-12953642:(+)	0.0741	1.005 (1.002, 1.008)	0.000
ENSG00000147378.10	FATE1	chrX:151716035-151723194:(+)	0.0862	2.217 (1.518, 3.238)	0.000
ENSG00000113739.9	STC2	chr5:173314713-173329503:(-)	0.0895	1.016 (1.005, 1.027)	0.004
ENSG00000197467.12	COL13A1	chr10:69801931-69964275:(+)	0.0910	1.021 (1.013,	0.000

Ensemble ID	Gene	Chromosome location	Coefficient of lasso model	HR	pvalue
ENSG00000017483.13	SLC38A5	chrX:48458537-48470256:(-)	0.1003	1.029) 1.008 (1.003, 1.014)	0.002
ENSG00000136997.13	MYC	chr8:127735434-127741434:(+)	0.1635	1.003 (1.002, 1.005)	0.000
ENSG00000138028.13	CGREF1	chr2:27098889-27119115:(-)	0.2105	1.015 (1.009, 1.021)	0.000
ENSG00000241563.3	CORT	chr1:10449719-10451902:(+)	0.2504	1.026 (1.016, 1.036)	0.000

2   HR: Hazard Ratio; CI: Confidence Interval

Univariate and multivariate Cox regression analyses genes signiture for patients with osteosarcoma in study cohort.

1   **Table3.** Univariate and multivariate Cox regression analyses genes signiture for patients
2   with osteosarcoma in study cohort.

Characteristics	Univariate		Multivariate	
	HR	p.value	HR	p.value
Gender (Female vs Male)	0.666 (0.317, 1.399)	0.283		
Age (<18 vs >=18)	0.909 (0.345, 2.393)	0.846		
Recurrence (No vs Yes)	19.348 (4.589, 81.563)	< 0.001	5.374 (1.183, 24.404)	0.029
Primary tumor site (Arm/Hand OR Pelvis vs Leg/Foot)	0.487 (0.168, 1.413)	0.185		
Specific tumor site (Femur vs Tibia or others)	0.642 (0.309, 1.337)	0.236		
riskscore	13.915 (7.067, 27.398)	< 0.001	9.869 (4.663, 20.887)	< 0.001

Relationship between risk score of the 20-marker-based prognostic classifier with os and clinical characteristics in the training set

1   Table 4 Relationship between risk score of the 20-marker-based prognostic classifier with os and clinical

2   characteristics in the training set

Characters	Level	Low risk	High risk	P value
n		42	42	
Gender	Female (%)	16 (38.1)	21 (50.0)	0.379
	Male (%)	26 (61.9)	21 (50.0)	
Age	<18 (%)	32 (76.2)	34 (81.0)	0.791
	>=18 (%)	10 (23.8)	8 (19.0)	
Overall survival	Alived (%)	42 (100.0)	13 (31.0)	<0.001
	Dead (%)	0 (0.0)	29 (69.0)	
Recurrence	No (%)	32 (76.2)	13 (31.0)	<0.001
	Yes (%)	10 (23.8)	29 (69.0)	
Metastasis	No (%)	37 (88.1)	26 (61.9)	0.011
	Yes (%)	5 (11.9)	16 (38.1)	
Primary tumor site	Arm/Hand OR Pelvis (%)	3 (7.1)	5 (11.9)	0.713
	Leg/Foot (%)	39 (92.9)	37 (88.1)	
Specific tumor site	Femur (%)	13 (31.0)	25 (59.5)	0.015
	Tibia or others (%)	29 (69.0)	17 (40.5)	