Identification of the prognostic Enhancer RNA in bladder cancer bone metastasis

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Research

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Abstract

Background

Bladder urothelial carcinoma (BLCA), one of the most common urinary system malignancies, has a high morbidity and metastasis rate. The enhancer RNAs (eRNAs), are derived from enhancer regions and contribute to enhancer function, whereas their roles in BLCA tumorigenesis and bone metastasis are still unclear.

Method

The RNA sequencing data of patients with BLCA were retrieved from The Cancer Genome Atlas (TCGA) databases and the normalized gene expression profile of eRNA in BLCA (annotated by Ensemble ID) were downloaded from the eRic database. The differential expression eRNAs and genes were evaluated and identified between primary BLCA with and without bone metastasis. The prognostic bone metastasis-specific eRNAs (PBMSEs) were screened by Lasso regression, and based on them, the risk scores were identified and predict models were constructed. Their accuracy were tested by the area under the curve (AUC) of the receiver operator characteristic (ROC) curve. Additionally, in order to explore the bone metastasis-specific regulation network, the relationship among key PBMSEs, differentially expressed target genes of eRNAs and signaling pathways were identified by Pearson correlation analysis.

Results

A total of 832 eRNAs were detected, with 37 PBMSEs annotated by Ensemble ID in univariate Cox regression analysis. Based on them, the first prediction model was constructed with the AUC of 0.920. Besides, we uncovered 166 DEGs between primary BLCA with and without bone metastasis. A total of 23 eRNAs and 109 target genes annotated by official gene symbol were uncovered in which31 PBMSEs and target genes revealed prognostic. Based on them, another prediction model was constructed with the AUC of 0.742. Moreover, we identified EMP1 as a PBMSE which regulated the target genes of APOLD1 and GPRC5A, and tumorigenesis-related pathways of KRAS signaling, inflammatory response, IL2-stat5 signaling and epithelial mesenchymal transition (EMT).

Conclusion

Our study identifies eRNAs as reliable indexes for prognosis and bone metastasis prediction in BLCA patients and provides well-applied prediction models for them. The bone metastasis-specific regulation network was also explored in which PBMSE (EMP1) could mediate target genes (APOLD1, GPRC5A) and tumorigenesis-related pathways (KRAS signaling, inflammatory response, EMT) in the tumorigenesis and bone metastasis of BLCA.

Introduction

Bladder urothelial carcinoma (BLCA) is a common malignant tumor in urinary system, with a high mortality andmetastasisrate[1,2]. Although surgical treatment and radiotherapy can provide favorable prognosis for localized lesions, they fail to cure advanced BLCA[3,4]. With regard to metastatic BLCA, bone is a common metastatic site, and the osteolytic destruction often results in the skeletal-related events (SREs), such as local pain, pathologic bony fracture and spinal cord compression[5]. It is reported that about 50% of BLCA patients die from tumor distant metastasis [6,7]. In this regard, tumor distant metastasis, especially bone metastasis, has become a key cause inducing poor prognosis of BLCA patients. In order to improve the overall survival (OS), there is a pressing need to explore the potential mechanism of its tumorigenesis and bone metastasis, thereby identifying the prognostic biomarkers and therapeutic targets.

As the important distal genomic elements, enhancers take part in the regulation of target gene expression by forming spatial chromatin loops with target promoters[8]. The malfunction of enhancers may induce genetic and epigenetic alterations, and subsequently drive tumorigenesis and recurrence in many cancers[9,10]. Enhancer RNAs (eRNAs), a type of noncoding RNAs transcribed from the enhancer, help to explore the mechanisms of enhancers and also participate in cancer development[11]. Its expression is also various in different tumor types and individual patients, thus may serve as potential diagnosis markers or therapeutic targets[12].

In order to explore the roles of eRNAs in regulating bone metastasis in BLCA, RNA-seq data and clinical information of BLCA samples were obtained from The Cancer Genome Atlas (TCGA) databases in this study. The differential expressed genes (DEGs) and eRNA expression were identified between primary BLCA with or without bone metastasis. The prognostic bone metastasis-specific eRNAs (PBMSEs) were then explored in BLCA and based on them, the predict model was constructed. Furthermore, the regulatory network of PBMSEs, differentially expressed target genes of eRNAs and downstream signaling pathways were also explored to provide the potential mechanism in BLCA bone metastasis, along with prognostic biomarkers and therapeutic targets.

Method

Primary data extraction

In formats of Fragments Per Kilobase per Million (FPKM) and HT-seq count, RNA-seq data of 411 primary BLCA samples (199 primary BLCA diagnosed without bone metastasis and 11 primary BLCA diagnosed with bone metastasis during the follow-up period) and 19 normal solid tissue samples were downloaded from The Cancer Genome Atlas (TCGA)(https://tcga-data.nci.nih.gov). Phenotypes of demographics data (age at diagnosis, race and gender), tumor information (histologic grade, AJCC clinical stage, TNM classification and bone metastasis diagnosis) and endpoint data (OS status and OS time) were also extracted in Xtensible Markup Language (XML) files from the database.

The eRNA expression matrix extraction

In the current study, the normalized gene expression profile of eRNA in BLCA (annotated by Ensemble ID) were downloaded from the eRic (enhancer RNA in cancers)database (https://hanlab.uth.edu/eRic/)[11]. In addition, the official gene symbol of each eRNA was identified by ChIPseeker packageaccordingtothelocationinhg38 genome[13].

Differential expression analysis and functional enrichment analysis

The DEGs analysis was firstly performed by limma and edgeR algorithm in eRNA and whole transcriptome, with the screening criteria of |log2 Fold Change (FC)| > 1.0 and False Discovery Rate (FDR) value < 0.05 [14,15]. The DEGs were identified between 199 primary BLCA diagnosed without bone metastasis and 11 primary BLCA diagnosed with bone metastasis during follow-up period based on the above-mentioned criteria. In additional, the functional enrichment analysis including Gene Oncology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) items were conducted to illuminate the biological processes (BPs), cellular components (CCs), molecular functions (MFs) and disease-related signaling pathways that DEGs enriched.[16]

Identification of prognostic bone metastasis-specific eRNAs (PBMSEs) in BLCA

The differential expression eRNAs (DEEs) were integrated into the univariate Cox regression analysis and DEEs annotated by Ensemble ID with P < 0.01 in univariate Cox regression analysis were defined as PBMSEs. Then, all PBMSEs were included in the multivariate Cox analysis. In terms of model diagnosis, the discrimination of the multivariate model was illustrated by the receiver operator characteristic (ROC) curve. Similarly, DEEs annotated by official gene symbols were also analyzed by the same process to identify PBMSEs. In the end, the key PBMSEs were reported in official gene symbols in this study.

Calculation of the riskscore (RS) and independent prognosis analysis

The formula of the multivariate Cox model (as followed) was used to calculate the risk score (RS) for each BLCA patient.

In the formula, “m” represented the number of each patient diagnosed with BLCA; “n” represented the number of PBMSE in the multivariate model; and “β” represented the coefficient of each PBMSE in the multivariate model. All BLCA patients were divided into the high-risk group and low-risk group with the median of the RS. Besides, the independent prognosis value of the RS in BLCA was evaluated by Kaplan-Meier survival analysis, univariate Cox analysis and multivariate Cox analysis corrected by demographics and clinical information.

The construction of the bone metastasis-specific regulation network including PBMSEs, differentially expressed target genes of eRNAs and signaling pathways

The target gene list of eRNA was extracted from the eRic (enhancer RNA in cancers)database (https://hanlab.uth.edu/eRic/)[11].Then, the 50 hallmarks of cancer gene sets were retrieved from the

Molecular Signatures Database (MSigDB) v7.1 (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp) [17]. Absolute quantification of the 50 hallmark of cancer gene sets in all samples were quantifiedas continuous variables by Gene Set Variation Analysis (GSVA)[18]. The co-expression analysis was performed among PBMSEs, differentially expressed target genes of eRNAs and absolute quantification of 50 hallmarks of cancer. Interaction pairs between PBMSEs and target genes with |correlation coefficient|> 0.80 and P value < 0.05 along with interaction pairs between PBMSEs and hallmarks of cancer with |correlation coefficient|> 0.30 and P value < 0.05 were used to construct the regulation network.

Statistics analysis

In this study, only two-sided P value < 0.05 was considered as statistically significant for all analysis process. The R software (www.r-project.org; version 3.6.1; Institute for Statistics and Mathematics, Vienna, Austria) were used for all statistics analysis processes.

Results

Differential expression analysis and univariate Cox regression analysis of eRNAs

All analysis process of the current study was summarized in the flowchart (Figure 1). A total of 832 eRNAs were detected in BLCA. Besides, 818 upregulated eRNAs (annotated by Ensemble ID) were identified as DEEs between 199 primary BLCA diagnosed without bone metastasis and 11 primary BLCA diagnosed with bone metastasis. The top 50 DEEs were illustrated in the heatmap (Figure 2A) and all of the DEEs were presented in the volcano plot (Figure 2B).

The DEEs in format of Ensemble ID and official gene symbol were integrated into the univariate Cox regression analysis. A total of 37 eRNAs annotated by Ensemble ID with P < 0.01 were identified in univariate Cox regression analysis and defined as prognostic bone metastasis-specific eRNAs (PBMSEs). All of them were integrated into multivariate Cox model (Figure 2C). Besides, when the cut off of P-value was set as 0.05, 91 PBMSEs were found.

Identification of RS and independent prognosis analysis

The RS for each BLCA patient was calculated by the formula described in the methodology. The distribution of RS among all BLCA patients were shown by the risk line and risk scatterplot (Figure 3A, B). The ROC illustrated decent discrimination of the multivariate Cox regression model with the AUC of 0.920 (Figure 3C). In addition, the Kaplan-Meier survival curve suggested that RS was a prognostic factor for BLCA patients (Figure 3D, P < 0.001).

The univariate and multivariate Cox model corrected by demographics and clinical information were constructed. The RS calculated by PBMSEs was proved to be an independent predictor for the prognosis of BLCA in both the univariate (HR = 147.989, 95%CI (29.216-749.617), P < 0.001, Figure 3E) and multivariate (HR = 1.057, 95%CI (1.038-1.076), P < 0.001, Figure 3F) Cox model.

The DEGs analysis and functional enrichment analysis

In the whole transcriptome DEG analysis, a total of 166 genes (8 downregulated genes and 156 upregulated genes) were identified asDEGs between primary BLCA diagnosed with and without bone metastasis. The heatmap and volcano plot were described in Figure 4A and B. The DEGs enriched in the GO term pointed to myelin sheath and vacuolar proton-transporting V-type ATPase complex (Figure 4C), while those enriched in KEGG term were associated with VEGF signaling pathway, vibrio cholerae infection and collecting duct acid secretion (Figure 4D).

The DEGs analysis and univariate Cox regression analysis of eRNAs annotated by official gene symbol

The official gene symbol of each eRNA was identified by ChIPseeker package according to the location in hg38 genome. Then, only 23 eRNAs (one downregulated gene and 22 upregulated genes) annotated by official gene symbol were found as PBMSEs between primary BLCA diagnosed with and without bone metastasis. The heatmap and volcano plot were presented in Figure 5A and B.A total of 23 eRNAs and 109 target genes annotated by official gene symbol were found as DEGs in which a total of 31 PBMSEs and target genes were shown to have prognostic values and integrated into multivariate Cox model (Figure 5C).

Identification of PBMSEs annotated by official gene symbol and independent prognosis analysis

The RS for each BLCA patient was calculated based on the multivariate Cox model including PBMSEs and target genes. The distribution of RS among all BLCA patients were shown in the risk line (Figure 6A) and risk scatterplot (Figure 6B). The ROC illustrated an acceptable discrimination in the multivariate Cox regression model with the AUC of 0.742 (Figure 6C). Moreover, the Kaplan-Meier survival curve suggested that RS had prognostic value for BLCA patients (Figure 6D, $P < 0.001$).

The univariate and multivariate Cox model were constructed after the correction by demographics and clinical information. The RS calculated by PBMSEs and target genes (annotated by official gene symbol) was shown as an independent factor for predicting prognosis of BLCA in both univariate (HR = 22.086, 95%CI (5.166-94.419), $P < 0.001$, Figure 6E) and multivariate (HR = 1.235, 95%CI (1.071-1.425), $P < 0.001$, Figure 6F) Cox model.

The construction of bone metastasis-specific regulation network including PBMSEs, differentially expressed target genes of eRNAs and signaling pathways

A total of 29 differentially expressed hallmarks of cancer gene sets were identified between primary BLCAs and normal solid tissue samples. They were presented in the heatmap (Figure 7A), volcano plot (Figure 7B) and bar plot (Figure 7C). Hedgehog signaling, myogenesis, KRAS signaling, angiogenesis, inflammatory response, IL2-stat5 signaling, epithelial mesenchymal transition (EMT) and IL6-JAK-stat3 signaling were highly enriched in the BLCA.

Next, point to point co-expression analysis were conducted among these 18 PBMSEs, 109 target genes and absolute quantification of 50 hallmarks of cancer. A total of 155 co-expression interaction pairs between PBMSEs and target genes, along with 38 co-expression interaction pairs between target genes (some target genes of eRNAs were themselves) and hallmarks of cancer, were used to construct the bone metastasis-specific regulation network (Figure 8A). Furthermore, seven PBMSEs were discovered which not only had a consistent prognostic value in format of Ensemble ID and official gene symbol but also had target genes co-expressed with downstream pathways (Figure 8B). Eventually, EMP1 was identified as the PBMSEs with the highest co-expression patterns and prognostic value (Figure 8C, D). It could regulate the target genes, such as APOLD1 and GPRC5A, and tumorigenesis-related pathways, such as KRAS signaling, inflammatory response, IL2-stat5 signaling and EMT.

Discussion

BLCA is one of the most common urinary system malignancies. Generally, patients with metastatic BLCA, especially bone metastasis, often have a poor prognosis[19]. Thus, early diagnosis and targeted therapy for metastatic BLCA are pressing needs in clinic. The eRNAs, derived from enhancer regions, may contribute to enhancer function and regulate the tumorigenesis and metastasis[20]. The identification of eRNAs may provide biomarkers for early diagnosis and therapeutic targets for clinical treatment[21]. In this study, we uncovered key PBMSEs and based on them, the risk scores were identified, along with the constructed prediction models. Both of them could well predict the prognosis of BLCA patients. Besides, we also built the bone metastasis-specific regulation network and found that EMP1, identified as a key PBMSEs, could enhance by its EMP1 gene and regulate the tumorigenesis and bone metastasis in BLCA.

As distal genomic elements, enhancers can provide DNA binding motifs to recruit transcription factors (TFs) and regulate target genes[12]. The enhancer malfunction may induce genetic and epigenetic alterations, which in turn activate oncogene expression and drive tumorigenesis. Thus, eRNAs play critical roles in gene regulation in the cancer initiation and progression[22,23]. However, how the eRNAs regulate the tumorigenesis and bone metastasis of BLCA, along with its roles in prognosis prediction is still unknown. In this study, we identified the key PBMSEs in the bone metastasis of BLCA and uncovered that the risk scores based on key PBMSEs were important biomarkers for predicting the prognosis of BLCA patients.

Based on the key PBMSEs, we constructed two prediction models and both of them achieved a good accuracy and applicability (AUC: 0.920 and 0.742). In clinic, the prediction model may help oncologists to make therapeutic strategy. Therefore, many previous studies have explored the biomarkers and predict the prognosis of patients with BLCA[24–26]. In these prediction models, various statistical methods, such as deep learning, Cox regression and LASSO regression analysis, have been used and many prognostic factors have been included, such as clinical information (age, clinical stage and metastasis), laboratory examination (inflammatory indicators); molecular features (competing endogenous RNA, immune infiltration) [24,25,27–29]. Despite the personalized risk factors have a good application in predicting the

prognosis of BLCA, none of them included the eRNA signatures and PBMSEs. Thus, our present study is a good supplemental to the existing prediction models for BLCA patients.

In order to explore the potential mechanism of BLCA bone metastasis, we constructed the bone metastasis-specific regulation network of PBMSEs, target genes and hallmarks of cancer. We identified EMP1 as a PBMSE with the highest co-expression patterns and prognostic value. The eRNA of EMP1 was found within the mRNA region and activated itself (EMP1) in BLCA bone metastasis. EMP1 belongs to the peripheral myelin protein 22-kDa (PMP22) gene family and has a high degree of domain structure homology with EMP2 and EMP3[30]. Generally, EMP1 is downregulated at the primary tumor site, whereas it may lead to treatment resistance and is related to poor prognosis[31,32]. In addition, EMP1 also promotes tumor metastasis by enhancing cell migration and EMP2 enhances tumor growth by promoting angiogenesis [33,34]. In this study, EMP1 was also found to regulate the target genes, such as APOLD1 and GPRC5A, and tumorigenesis-related pathways, such as KRAS signaling, inflammatory response, IL2-stat5 signaling and EMT.

APOLD1, an endothelial cell early response protein, takes part in regulation of endothelial cell signaling and responds to different stimuli such as cytokines, growth factors and stress[35]. It was mapped to 12p13.1, the susceptibility locus for testicular germ cell tumor, indicating its pathogenesis roles in this tumor [36]. GPRC5A, a retinoic acid inducible gene, plays dual roles in tumorigenesis. It is highly expressed in normal lung tissues and GPRC5A-knockout mice develop spontaneous lung cancer[37]. On the other hand, its elevated expression is found in various tumor samples and related to tumor growth and colony formation[38,39]. In bladder cancer, GPRC5A serves as a surface protein in bladder cancer stem cells and drives cancer stem cells self-renewal and metastasis [40,41].

KRAS signaling, inflammatory response, IL2-stat5 signaling and EMT are common cancer- and metastasis-associated pathways in BLCA. K-Ras-ERK1/2 signaling pathway was reported to induce tumorigenesis of BLCA via inhibition of H1.2 phosphorylation at T146[42]. In addition, inflammation was also associated with BLCA. Cytokine can induce the imbalances of tumor-infiltrating cytotoxic cells and subsequently boost BLCA growth[43]. Targeting the inflammation in the tumor microenvironment is a feasible method to suppress BLCA metastasis [44,45]. Moreover, EMT is an important process in the tumor metastasis. In BLCA, circular RNA PRMT5 induces EMT and promotes metastasis[46]. Besides, TGF-β-induced transgelin can also promote BLCA metastasis via EMT[47].

Although this study is the first one to identify the roles of eRNAs in the BLCA tumorigenesis and bone metastasis, it still possessed some limitations that warrant consideration. Firstly, all the samples involved in this study are from America, and thus we are not quite sure about the applicability of prediction model in European and Asian. Second, this proposed prediction model has not been verified by external databases, and thus our future work is validating it by our own data and other public database. Third, the potential mechanism in this study is based on bioinformation analysis and has not been verified by

molecular and animal experiments. Thus, we plan to verify these potential mechanisms via molecular experiments.

Conclusion

Our study identifies eRNAs as reliable indexes for prognosis and bone metastasis prediction in BLCA patients and provides well-applied prediction models for them. The bone metastasis-specific regulation network was also explored in which PBMSE (EMP1) could mediate target genes (APOLD1, GPRC5A) and tumorigenesis-related pathways (KRAS signaling, inflammatory response, EMT) in the tumorigenesis and bone metastasis of BLCA.

Declarations

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethical Review Committee Statement (IRB approval)

The Ethics Committee of the First Affiliated Hospital, Jinan University approved this study.

Data Availability Statement

The data were also available in the TCGA-BLCA program (https://www.cancer.gov/tcga,).

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Figures

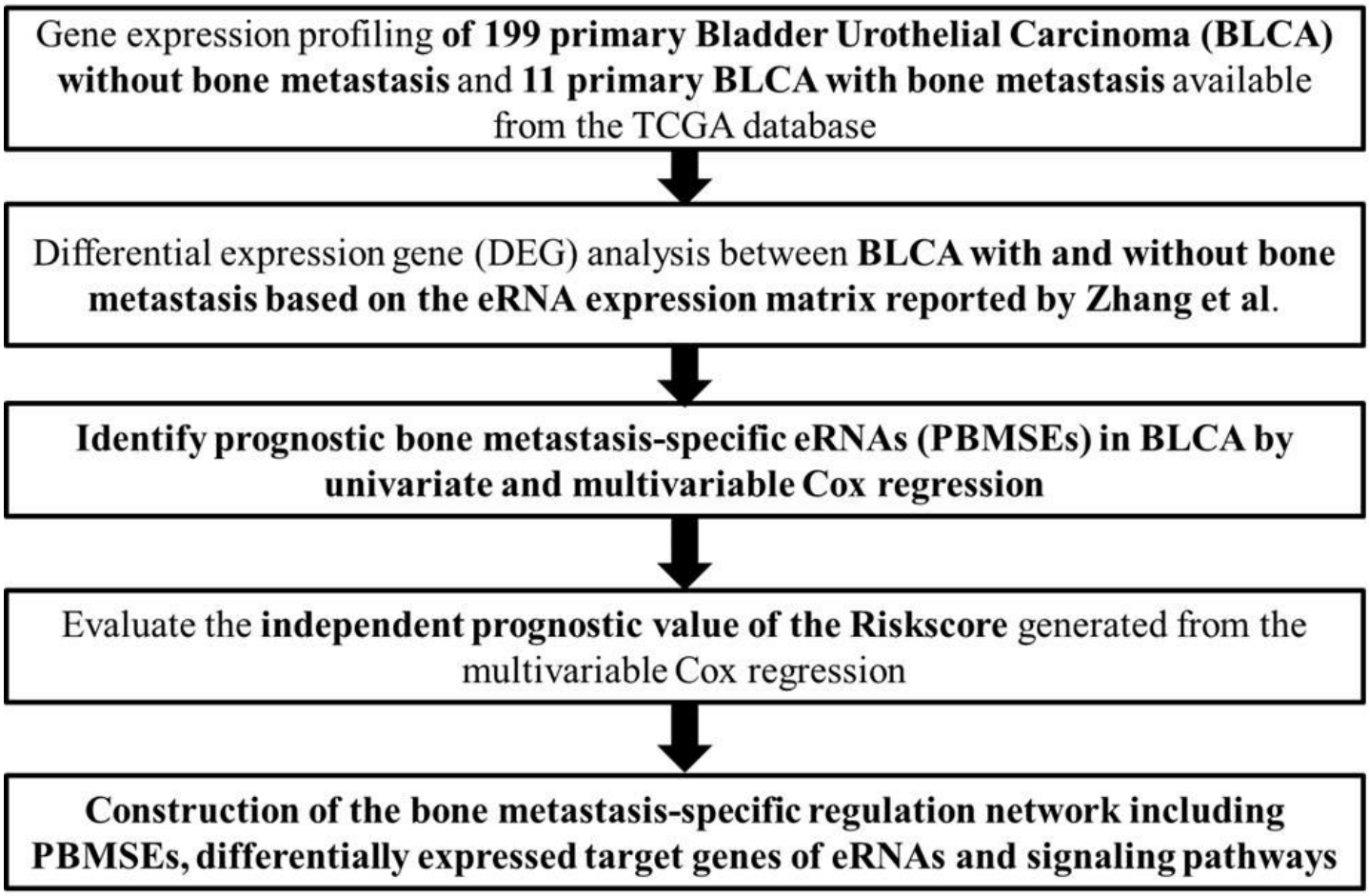

Figure 1

The flowchart of all analysis processes of the current study.

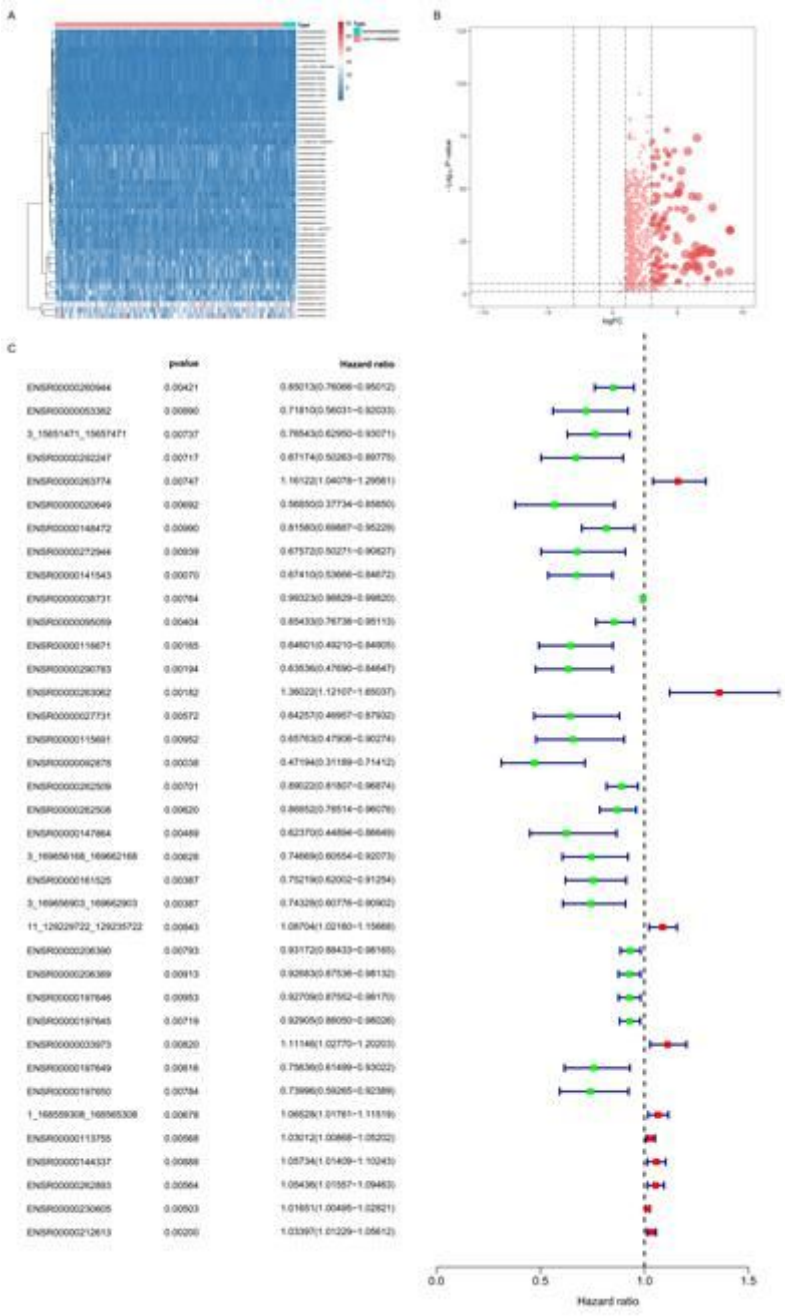

Figure 2

The results of differential expression genes analysis and univariate Cox regression analysis of eRNAs annotated by Ensemble ID A total of 832 eRNAs were detected in BLCA. 818 upregulated eRNAs (annotated by Ensemble ID) were identified as DEGs between 199 primary BLCA diagnosed without bone metastasis and 11 primary BLCA diagnosed with bone metastasis (A-B, top 50 DEGs were illustrated in the heatmap).The DEEs in format of Ensemble ID and official gene symbol were integrated into the univariate Cox regression analysis, respectively. DEEs annotated by Ensemble ID with $P < 0.01$ in univariate Cox regression analysis were defined as prognostic bone metastasis-specific eRNAs (PBMSEs). The results illuminated that 37 eRNAs (there were 91 PBMSEs when set the cut off P-value as

0.05) annotated by Ensemble ID were identified as PBMSEs and integrated into multivariate Cox model (C).

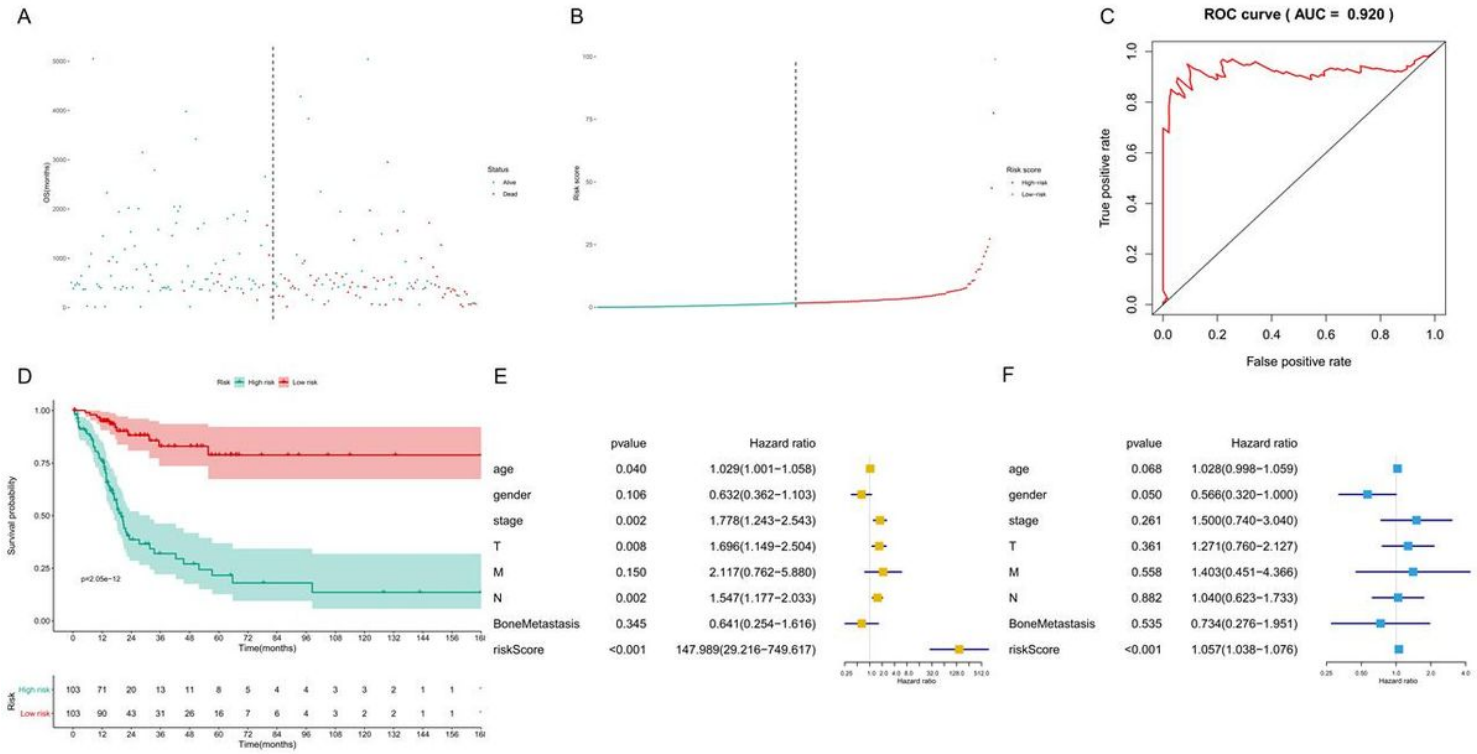

Figure 3

Identification of PBMSEs annotated by Ensemble ID and independent prognosis analysis The RS for each BLCA patient was calculated by the formula described in the methodology. The distribution of RS among all BLCA patients were shown by the risk line and risk scatterplot (A-B). The ROC (AUC = 0.920) illustrated decent discrimination of the multivariate Cox regression model (C). And the Kaplan-Meier survival curve suggested RS had prognostic value for BLCA patients (D, P < 0.001). In univariate (HR = 147.989, 95%CI (29.216-749.617), P < 0.001) (E) and multivariate (HR = 1.057, 95%CI (1.038-1.076), P < 0.001) (F) Cox model corrected by demographics and clinical information, RS calculated by PBMSEs (annotated by Ensemble ID) was shown to be an independent factor for predicting prognosis of BLCA.

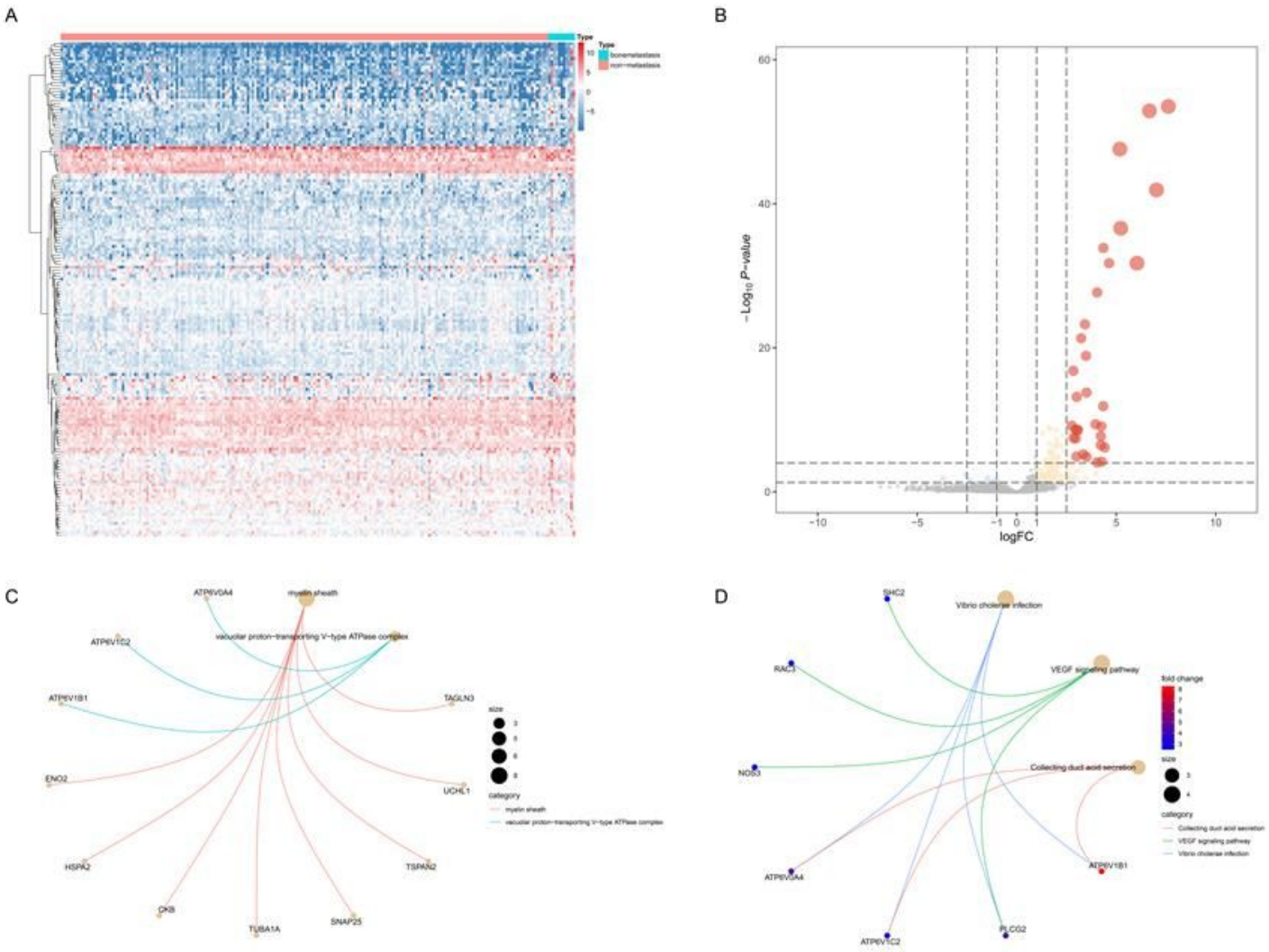

Figure 4

The results of differential expression genes analysis between primary BLCA diagnosed with and without bone metastasis and functional enrichment analysis. In whole transcriptome DEG analysis, a total of 166 genes (8 downregulated genes and 156 upregulated genes) were identified as DEGs between primary BLCA diagnosed with and without bone metastasis (A-B). Most significant enrichment items of GO terms and KEGG pathways for DEGs were myelin sheath and VEGF signaling pathway (C-D).

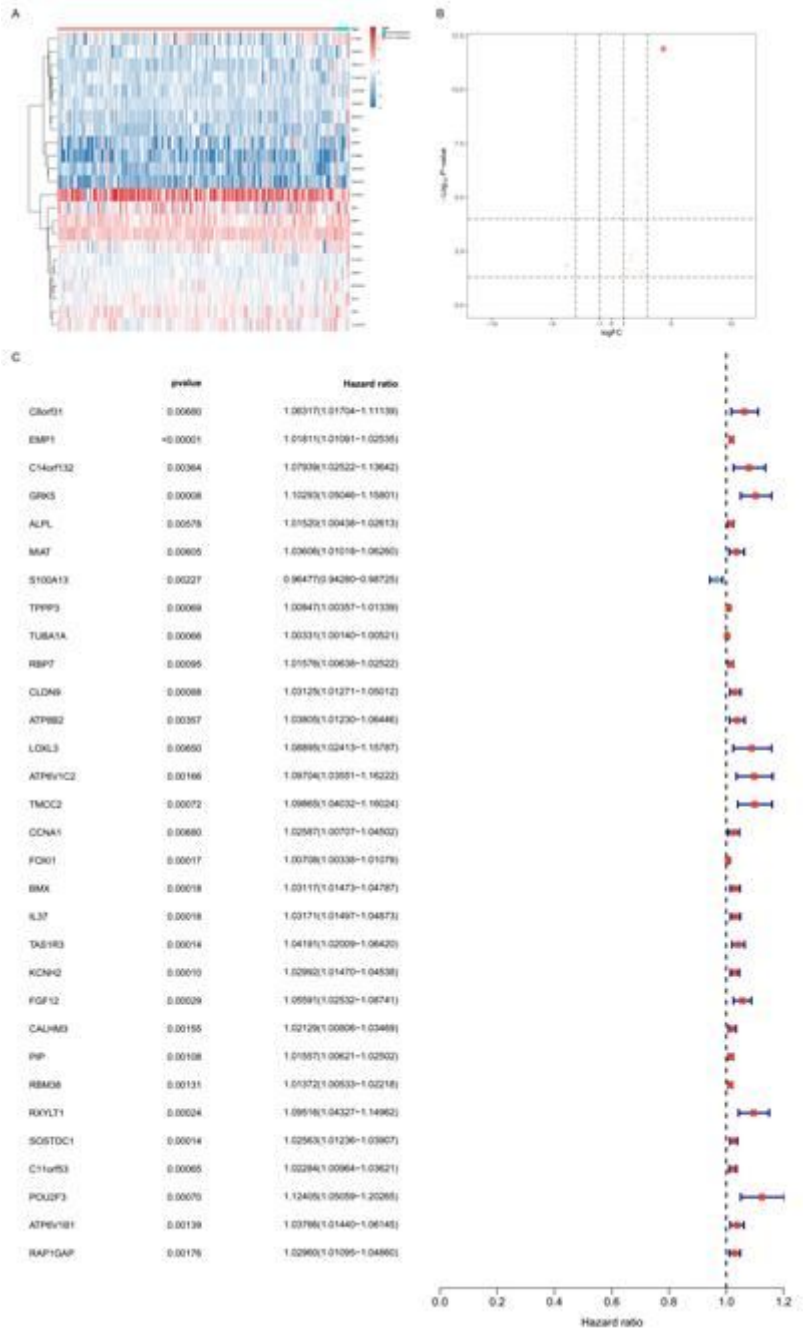

Figure 5

The results of differential expression genes analysis and univariate Cox regression analysis of eRNAs annotated by official gene symbol The official gene symbol of each eRNA was identified by ChIPseeker package according to the location in hg38 genome. Then, only 23 eRNAs(one downregulated gene and 22 upregulated genes) annotated by official gene symbol were found as DEGs between primary BLCA diagnosed with and without bone metastasis (A-B). 23 eRNAs and 109 target genes annotated by official gene symbol were found as DEGs in which a total of 31 PBMSEs and target genes were shown to have prognostic values and integrated into multivariate Cox model (C).

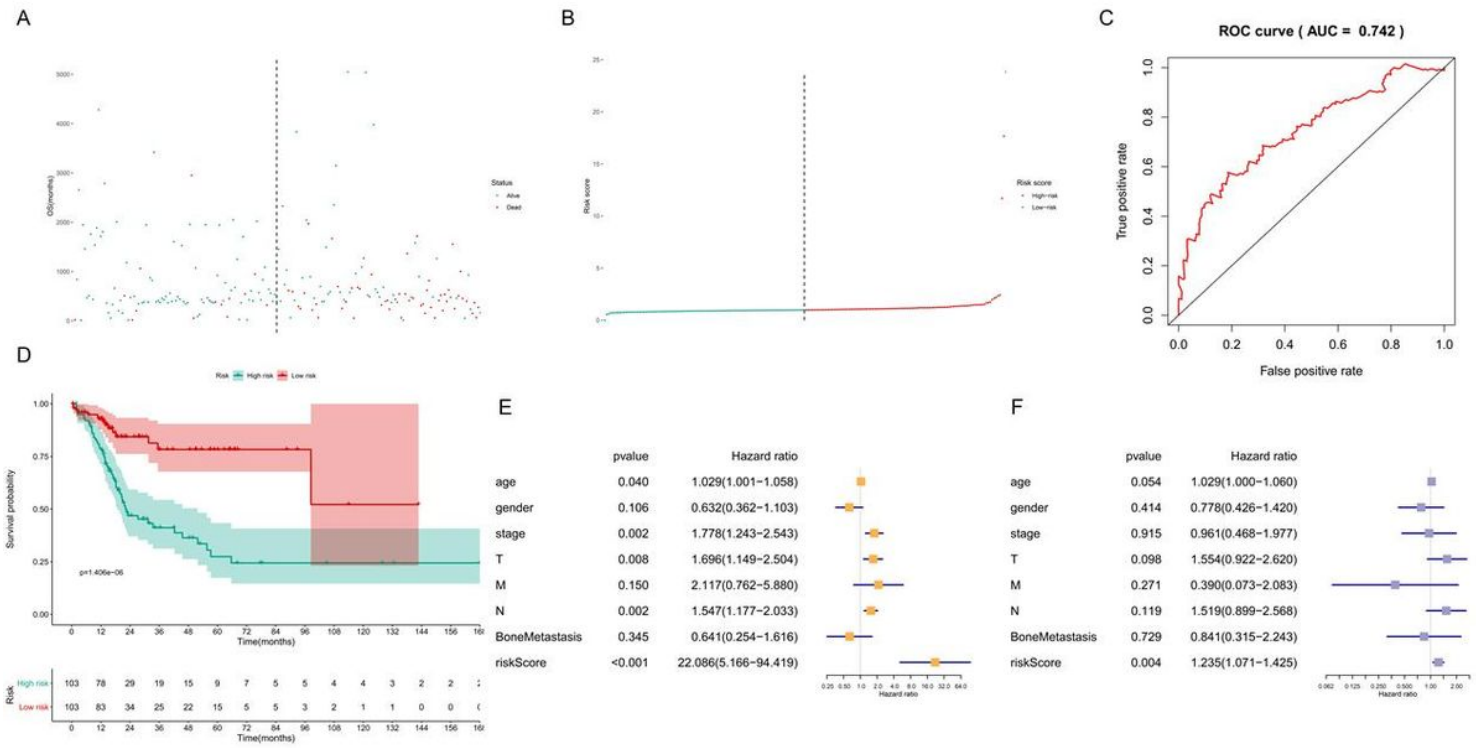

Figure 6

Identification of PBMSEs annotated by official gene symbol and independent prognosis analysis Similarly, the RS for each BLCA patient was calculated based on the multivariate Cox model including PBMSEs and target genes. The distribution of RS among all BLCA patients were shown by the risk line and risk scatterplot (A-B). The ROC (AUC = 0.742) illustrated acceptable discrimination of the multivariate Cox regression model (C). And the Kaplan-Meier survival curve suggested RS had prognostic value for BLCA patients (D, P < 0.001). In univariate (HR = 22.086, 95%CI (5.166-94.419), P < 0.001) (E) and multivariate (HR = 1.235, 95%CI (1.071-1.425), P < 0.001) (F) Cox model corrected by demographics and clinical information, RS calculated by PBMSEs and target genes (annotated by official gene symbol) was also shown to be an independent factor for predicting prognosis of BLCA.

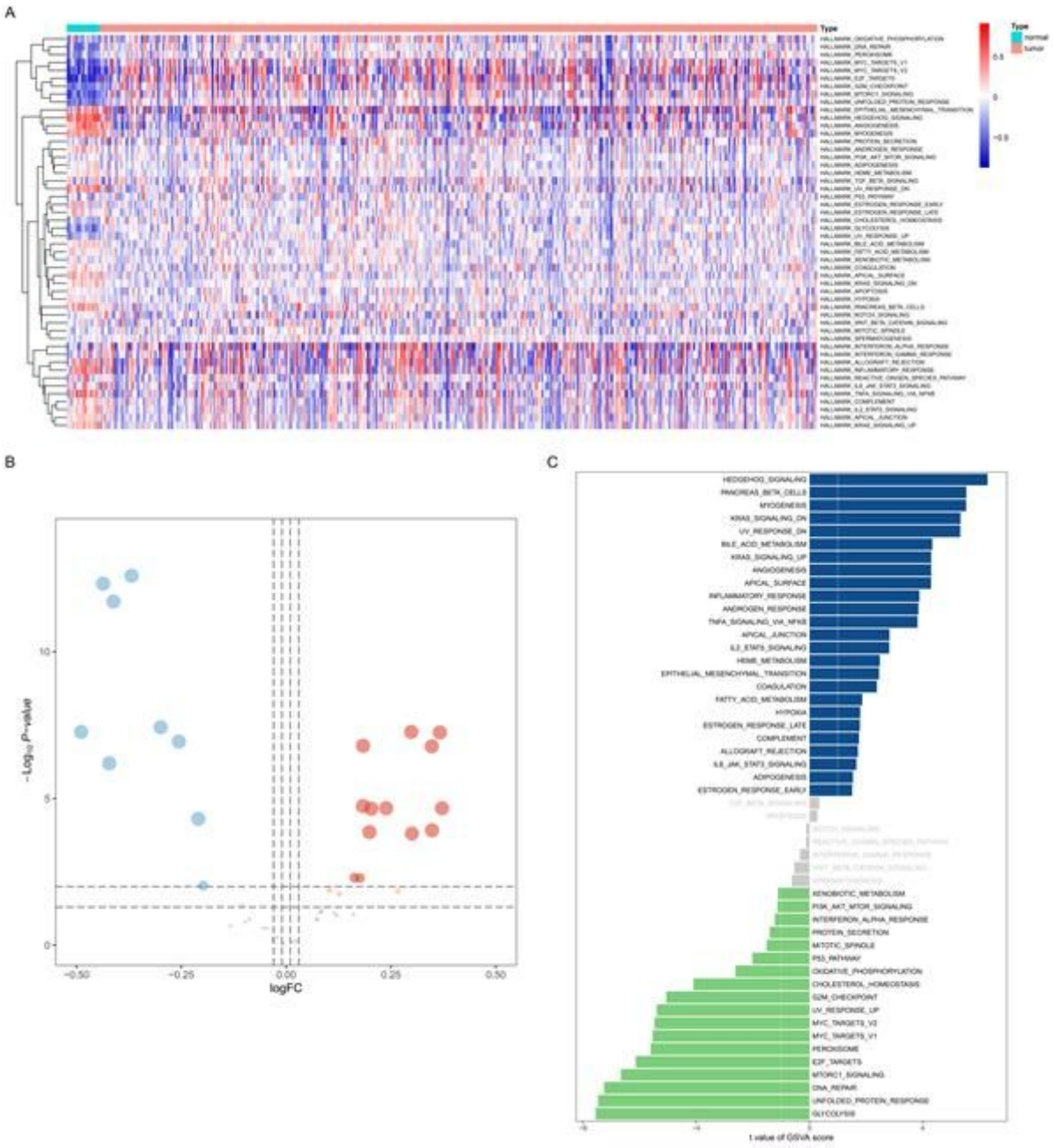

Figure 7

Gene Set Variation Analysis (GSVA) The heatmap (A), volcano plot (B) and bar plot (C) summarized 29 differentially expressed hallmarks of cancer gene sets between primary BLCAs and normal solid tissue samples.

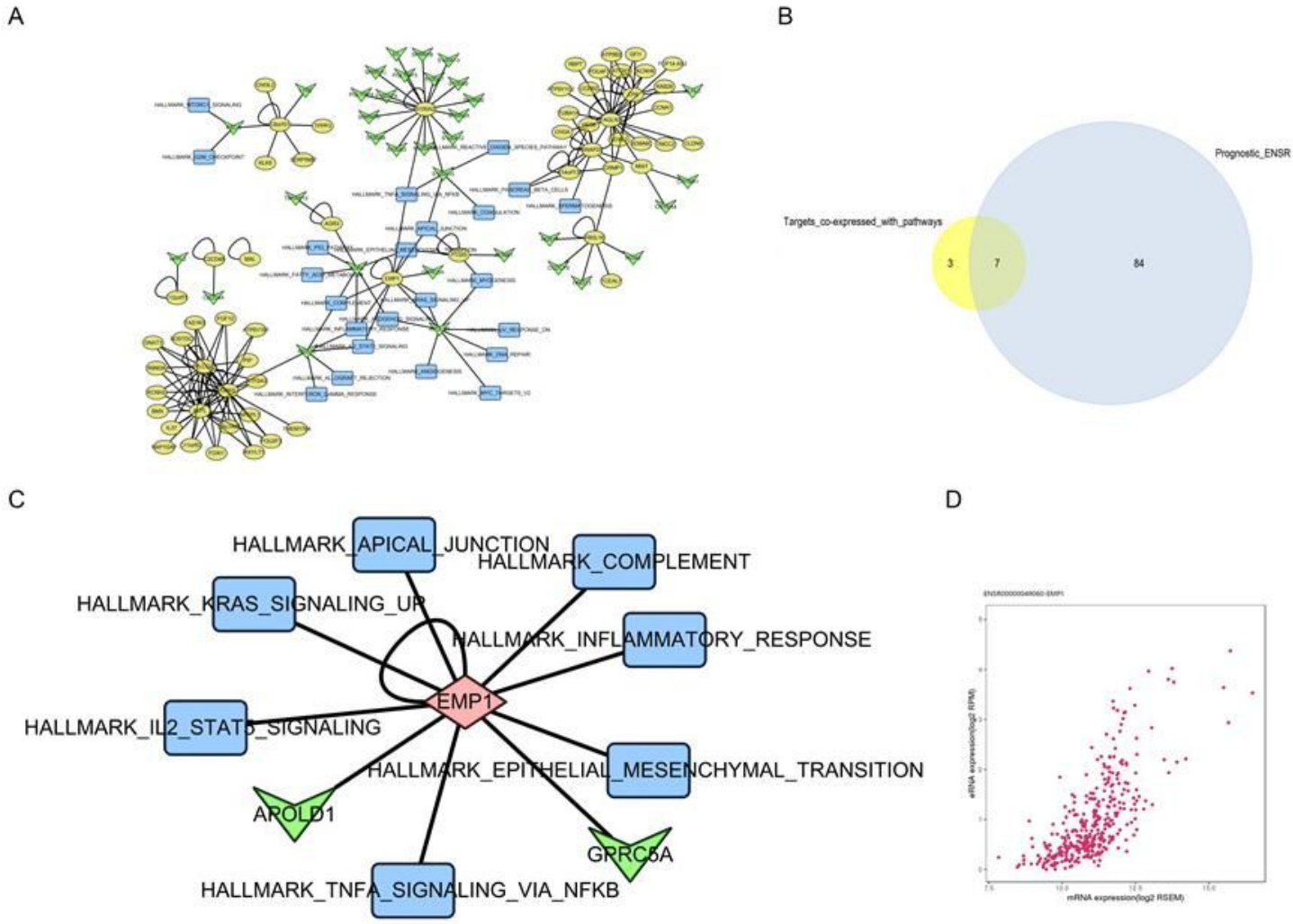

Figure 8

Construction of the bone metastasis-specific regulation network including PBMSEs, differentially expressed target genes of eRNAs and signaling pathways Point to point co-expression analysis were conducted among these 18 PBMSEs, 109 target genes and absolute quantification of 50 hallmarks of cancer. 155 co-expression interaction pairs between PBMSEs and target genes and 38 co-expression interaction pairs between target genes (some target genes of eRNAs were themselves) and hallmarks of cancer passed the criteria and were used to construct of the bone metastasis-specific regulation network (A). Furthermore, only seven PBMSEs not only had the consistent prognostic value in format of Ensemble ID and official gene symbol but also had target genes co-expressed with downstream pathways(B). Eventually, EMP1 was identified as the PBMSEs with the highest co-expression patterns and prognostic value (C-D).