

Discovery and validation of immune-related long non-coding RNA biomarkers associated with prognosis in hepatocellular carcinoma

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Abstract

Background : Hepatocellular carcinoma (HCC) is one of the most common clinical malignant tumors, resulting in high mortality and poor prognosis. Studies have found that LncRNA plays an important role in the onset, metastasis and recurrence of hepatocellular carcinoma. The immune system plays a vital role in the development, progression, metastasis and recurrence of cancer. Therefore, immune-related lncRNA can be used as a novel biomarker to predict the prognosis of hepatocellular carcinoma.

Methods : The transcriptome data and clinical data of HCC patients were obtained by using The Cancer Genome Atlas-Liver Hepatocellular Carcinoma (TCGA-LIHC), and immune-related genes were extracted from the Molecular Signatures Database (IMMUNE RESPONSE M19817 and IMMUNE SYSTEM PROCESS M13664). By constructing the co-expression network and Cox regression analysis, 13 immune-lncRNAs was identified to predict the prognosis of HCC patients. Patients were divided into high risk group and low risk group by using the risk score formula, and the difference in overall survival (OS) between the two groups was reflected by Kaplan-Meier survival curve. The time - dependent receiver operating characteristics (ROC) analysis and principal component analysis (PCA) were used to evaluate 13 immune -lncRNAs signature.

Results : Through TCGA - LIHC extracted from 343 cases of patients with hepatocellular carcinoma RNA - Seq data and clinical data, 331 immune-related genes were extracted from the Molecular Signatures Database , co-expression networks and Cox regression analysis were constructed, 13 immune-lncRNAs signature was identified as biomarkers to predict the prognosis of patients. At the same time using the risk score median divided the patients into high risk and low risk groups, and through the Kaplan-Meier survival curve analysis found that high-risk group of patients' overall survival (OS) less low risk group of patients. The AUC value of the ROC curve is 0.828, and principal component analysis (PCA) results showed that patients could be clearly divided into two parts by immune-lncRNAs, which provided evidence for the use of 13 immune-lncRNAs signature as prognostic markers.

Conclusion : Our study identified 13 immune-lncRNAs signature that can effectively predict the prognosis of HCC patients, which may be a new prognostic indicator for predicting clinical outcomes.

Background

Hepatocellular carcinoma (HCC) is the most common histological type of primary liver cancer [1]. Worldwide, HCC is one of the most common malignant tumors, the fourth leading cause of cancer death, and the sixth most common malignant tumor in the world [2]. Because of its strong invasion and high incidence of metastasis, hepatocellular carcinoma often has a poor prognosis, so early diagnosis and treatment have become the key to improve the survival rate of HCC patients. It is necessary to develop efficient and non-invasive markers for early diagnosis and prognosis.

The occurrence and development of hepatocellular carcinoma (HCC) is a complex process involving multiple genes and multiple pathways [3]. Long non-coding RNA (lncRNA) is a class of regulatory

macromolecular RNA discovered in recent years. Its transcripts were longer than 200nt. For a long time, lncRNA was considered as a meaningless transcriptional by-product [4]. Recent studies have found that lncRNA can regulate gene expression at transcriptional, post-transcriptional and epigenetic levels, and participate in many pathophysiological processes such as activation of proto-oncogenes, transcriptional activation and interference, binding and regulating the activity of corresponding proteins [5]. Current studies have shown that lncRNA plays an important role in the pathogenesis, metastasis and recurrence of hepatocellular carcinoma [6, 7].

The occurrence and development of hepatocellular carcinoma (HCC) is closely related to the medium stimulation in the microenvironment [8]. Hepatocellular carcinoma (HCC) is a complex ecosystem containing non-tumor cells (mainly immune-related cells), and the success of immune checkpoint suppression in solid tumors underscores the critical role of tumor microenvironment in cancer progression [9]. Therefore, immune-related lncRNAs can be used as novel biomarkers to predict the prognosis of hepatocellular carcinoma.

In this study, we through the The Cancer Genome Atlas - Liver Hepatocellular Carcinoma (TCGALIHCC) and the Molecular Signatures Database to collect the transcriptome data of HCC patients clinical data and immune related genes. Through the establishment of co-expression network and Cox regression analysis, immune-related lncRNAs that can predict the prognosis of HCC patients were identified, and use the risk score to HCC patients are grouped, multivariate Cox regression analysis confirmed that the identified lncRNA has the independent prognostic role. Time-dependent receiver operating characteristics (ROC) analysis and principal component analysis (PCA) were used to further predict their prognostic ability, and gene set enrichment analysis (GSEA) was used to help explain the underlying mechanism.

Materials And Methods

The patient cohort and lncRNA profiles were mined

Transcriptome data and clinical information of Hepatocellular Carcinoma patients can be extracted from The Cancer Genome Atlas - liver Hepatocellular Carcinoma (TCGA-LIHC) (<http://larssonlab.org/tcga-lncrnas/index.php>) [10, 11]. After evaluating the clinical data, 343 patients with hepatocellular carcinoma (HCC) were included in the study after excluding patients who were followed up for no more than 30 days (because they were likely to die from fatal complications other than HCC) or who had RNA-seq data but no clinical prognostic information [12, 13]. In order to identify the differential expression of lncRNA and mRNA between HCC and adjacent non-tumor tissues, the R/Bioconductor package "edgeR" was used to process the mRNA and lncRNA expression data [14]. Immune-related genes were extracted from the Molecular Signatures Database (IMMUNE RESPONSE M19817 and IMMUNE SYSTEM PROCESS M13664) [15]. Immune-related lncRNAs were identified by establishing a co-expression network. Univariate and multivariate Cox regression analysis was used to analyze the immune-related lncRNA ($p < 0.05$) related to the survival time of patients for model construction [16].

Validation of prognostic model and survival analysis

Immune-lncRNAs associated with prognosis were selected and the prognostic model was further constructed by multivariate Cox analysis. After the expression values of each specific gene were included, the risk score formula for each patient was constructed and weighted by its estimated regression coefficients in the multi-factor regression analysis. According to the risk score formula:

$$\text{Risk Score (patient)} = \sum_{n=1}^{\infty} \text{coefficient (lncRNA}_i) \times \text{expression (lncRNA}_i)$$

Here, lncRNA_i represents the prognostic lncRNA and expression (lncRNA_i) is the expression level of lncRNA_i for the patient. Regression coefficient of multivariate Cox analysis was denoted as coefficient (lncRNA_i) which represents the contribution of lncRNA_i for prognostic risk scores [17]. Patients with higher risk scores tend to have poorer survival. HCC patients can be assigned to high-risk or low-risk groups according to the median risk as the critical point. Overall survival differences between high-risk and low-risk groups were assessed using the Kaplan-Meier method and compared using the log-rank test [18].

Statistical analysis

In order to evaluate the independence of immune-related lncRNA signature from key clinical factors, multi-factor regression analysis and stratified analysis were used to examine the role of risk score in predicting patient prognosis, hazard ratio (HR) and 95% confidence interval (CI) were calculated by Cox analysis, and clinical correlation analysis was performed [19]. Survival prediction comparisons based on lncRNA signature and key clinical characteristics were performed using time-dependent receiver operating characteristics (ROC) analysis [20]. Based on principal component analysis (PAC) of immune-lncRNAs and whole genes, the prognostic accuracy of the identified immune-related lncRNAs was further verified [21].

Functional enrichment analysis

Through the Gene Set Enrichment Analysis (GSEA) of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO), the possible molecular mechanism of the prognosis difference between the high-risk group and the low-risk group was investigated [22, 23]. The genes were sequenced according to the degree of differential expression in the high-risk group and the low-risk group, and then the enrichment of the preset gene set was examined.

Results

The co-expression network was constructed to identify the immune-related lncRNAs signature

Transcriptome data of 374 Hepatocellular Carcinoma (HCC) and 50 adjacent normal tissue samples were extracted from The Cancer Genome Atlas liver Hepatocellular Carcinoma Dataset (TCGA-LIHC). RNA-seq data for HCC included 14142 lncRNAs and 19,658 mRNAs expression profiles. Transcriptome data and clinical data of a total of 343 HCC patients were included in our study according to the patients' follow-up time range of 30-3675 days and the exclusion of patients with rna-seq data but no clinical prognosis information. A total of 331 genes involved in immune-related pathways were extracted from the Molecular Signatures Database (IMMUNE RESPONSE M19817 and IMMUNE SYSTEM PROCESS M13664). Immune-related lncRNA was identified by constructing an immune co-expression network, and 542 lncRNAs were identified as immune-related ($P \leq 0.01$) (Table S1). The immune-related lncRNA expression was combined with the survival data, and 56 immune-related lncRNAs (Table S2) related to patient prognosis were identified by univariate Cox regression analysis and the forest map was drawn (Fig. 1). Using multi-factor Cox regression analysis, 13 immunity-related lncRNAs were further screened out for prediction model construction (Table 1), including AC080013.6, AC080013.6, AC019080.5, LINC01554, PTOV1-AS1, MIR210HG, MSC-AS1, AL049840.2, AC023157.3, AL031985.3, AC145207.5, AC010761.1, and Z83851.2.

Prognostic validation of 13 immune-related lncRNAs signature in HCC patients

In order to establish the focused expression characteristics of immune-related lncRNAs for survival prediction, the expression profiles of 13 immune-lncRNAs signatures with independent prognosis were selected to establish a multivariate Cox regression model to evaluate their relative predictive power. In the above multi-factor Cox analysis, the expression level of immune-related lncRNAs with independent prognosis and the weighted method of regression coefficient were used to characterize 13 immune-related lncRNAs signature. The estimation method of prognostic risk score was as follows: Risk score (patient) = $(0.4156 \times \text{expression value of AC080013.6} + 0.1793 \times \text{expression value of PRRT3-AS1} + 0.7371 \times \text{expression value of AC019080.5} + 0.1256 \times \text{expression value of LINC01554} + 0.9524 \times \text{expression value of PTOV1-AS1} + 0.2331 \times \text{expression value of MIR210HG} + 0.4169 \times \text{expression value of MSC-AS1} + 0.8936 \times \text{expression value of AL049840.2} + 0.8392 \times \text{expression value of AC023157.3} + 0.7777 \times \text{expression value of AL031985.3} + 0.5308 \times \text{expression value of AC145207.5} + 0.6430 \times \text{expression value of AC010761.1} + 0.5159 \times \text{expression value of Z83851.2})$. Calculate each of the prognosis of patients with risk score, according to the score of the median value, 343 patients were divided into high-risk and low-risk group (Fig. 2A), at the same time to calculate the risk score is associated with cancer death (Fig. 2B), the relationship between as shown in the heat map (Fig. 2C), LINC01554 expression level increased with the increase of risk score gradually reduce, the rest of the immune related lncRNA expression level gradually increased with the increase of the risk score. Kaplan-meier analysis was used to evaluate the difference in survival between the high-risk group and the low-risk group (Fig. 2D), and the results showed that the overall survival (OS) of the high-risk group was worse than that of the low-risk group.

Evaluation of 13 immune-related lncRNAs signature models

Univariate and multivariate Cox regression methods were used to determine whether 13 immune-related lncRNAs signature could be used as independent predictors of HCC patients. According to the results of univariate analysis, risk score was significantly correlated with prognosis. After multivariate adjustment using the above factors, the risk score remained a reliable and independent predictor of risk in the cohort ($P < 0.001$) (Table 2). Based on univariate and multivariate analyses, we constructed a forest graph (Fig. 3A, B) that included independent prognostic factors (risk score, age, gender, grade, stage and TMN staging). The prognostic effects of 13 immune-related lncRNAs signature was evaluated by time-dependent receiver operating characteristics (ROC) analysis and calculating the area under the ROC curve (AUC) (Fig.3C). The results showed that the AUC of 13 immune-related lncRNA signature was 0.828, showing higher specificity and sensitivity compared with other independent prognostic factors. At the same time, the clinical correlation between 13 immune-lncRNAs signature and T staging was analyzed (Fig.3D), and it was found that AC010761.1, AC023157.3, AC145207.5, AL031985.3, LINC01554, MIR210HG, and ptoV1-as1 were correlated with T staging. Through principal component analysis (PCA), based on the 13 immune related lncRNAs signature (Fig. 4A) and the gene expression profile (Fig. 4B) studies the difference between low risk and high risk groups, show that low risk and high risk groups are usually distributed in different directions, the risk gene can significantly HCC patients can be divided into two parts, also for the identification of 13 immune related lncRNAs signature as to provide evidence for the HCC patients prognosis.

Possible molecular mechanisms underlying the differential prognosis of HCC patients

Further executive function was performed by gene set enrichment analysis (GSEA) (select TOP6 for display), Gene Set Enrichment Analysis (GSEA) of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway indicated significant enrichment of immune phenotype (Table 3) (Fig. 5): Progesterone mediated oocyte maturation, Ubiquitin mediated proteolysis, Oocyte meiosis, Cell cycle, Inositol phosphate metabolism, Vasopressin regulated water reabsorption. Gene Set Enrichment Analysis (GSEA) of Gene Ontology (GO) indicated significant enrichment of immune phenotype (Table 4) (Fig. 6): Spliceosomal complex assembly, Chromatin remodeling, Peptidyl lysine modification, Regulation of generation of precursor metabolites and energy, Import into nucleus, Positive regulation of cell cycle phase transition. These findings provide evidence for further exploration of the possible molecular mechanisms underlying the prognostic differences in HCC patients.

Discussion

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in clinical practice and the third leading cause of cancer death, accounting for more than 80% of the incidence of liver cancer [24]. Hepatocellular carcinoma is characterized by strong invasiveness and high incidence of metastasis, resulting in high mortality and poor prognosis, with a 5-year survival rate of only 10%-30% [25, 26]. Early diagnosis and treatment have become the key to improve the survival rate of HCC patients. At present, common clinical markers of liver cancer include alpha fetoprotein (AFP) (desrcarboxy prothrombin (DCP)) (squamous cell carcinoma antigen-IgM complexes (SCCA-IgMCs), etc [27, 28]. Although these markers

have some specificity in the diagnosis of HCC, they are less sensitive for early diagnosis, and HCC is usually diagnosed at an advanced stage. Therefore, it is necessary to develop efficient and non-invasive new markers for early diagnosis and prognosis.

Tumor microenvironment (TME) is a complex integrated system of tumor cell growth [29, 30]. This microenvironment is composed of tumor constituent cells, endothelial cells, immune cells, fibroblasts and extracellular matrix [31]. The immune system plays an important role in the tumor microenvironment and plays a vital role in the development, progression, metastasis and recurrence of cancer [32]. Cancer cells and immune cells show metabolic reprogramming in the tumor microenvironment, which is closely related to immune cell function and edits tumor immunology [33]. The latest findings in immune cell metabolism offer broad prospects for clinical therapies to treat cancer and will be critical to the development of identifying biomarkers [9, 34].

LncRNAs are a class of RNAs that are longer than 200 nucleotides but do not encode proteins [35]. Studies have proved that its expression is tissue-specific, plays an important role in the life process, and participates in almost all aspects of cell life activities. It can participate in the regulation of cell proliferation, cell differentiation and other life activities at epigenetic level, transcriptional level and post-transcriptional level [36, 37]. Therefore, abnormal expression of lncRNA is closely related to the occurrence and development of many human diseases, including tumors [38, 39]. Many studies have proved that lncRNAs can regulate gene expression through interaction with proteins or RNA, thus regulating cell proliferation, apoptosis and metastasis and participating in the occurrence and development of HCC [40]. Differential expression of lncRNAs in tissues can distinguish between cancer tissues and adjacent tissues, indicating that lncRNAs in cancer tissues have a characteristic expression profile [41]. At present, the abnormal expression of many lncRNAs in hepatocellular carcinoma has been identified. In HCC tumor tissues, the up-regulation of lncRNAs such as H19 and HOTAIR is associated with distant metastasis, and the down-regulation of lncRNA such as MEG3 is associated with cell proliferation [42–44]. Since the abnormal expression of some lncRNAs has been proved to be closely related to cancer, the use of lncRNAs as a marker for cancer diagnosis and prognosis has also become a new research direction [45]. Currently, immune-related lncRNA has not been reported as a prognostic marker for HCC patients.

In this study, we obtained transcriptome information and clinical data of a HCC patient using TCGA-LIHC Database, and obtained immune-related genes through Molecular Signatures Database. Through the establishment of co-expression network and Cox regression analysis, 13 immune-related lncRNAs signature was identified to predict the prognosis of HCC patients. Risk score was used to distinguish HCC patients into high-risk group and low-risk group, and the differential expression of 13 immune-lncRNAs signature in the two groups was analyzed. Kaplan-Meier survival chart was used to analyze the difference in survival status between high-risk group and low-risk group. Using univariate and multivariate Cox regression analysis, to determine the 13 kinds of immune related lncRNA can serve as an independent predictor of HCC of patients, and use the time-dependent receiver operating characteristics (ROC) curve, AUC value of 0.828, to evaluate the immune - lncRNAs on overall survival of predictive value, through the

principal component analysis (PCA) has once again proven its predictive value. Gene set enrichment analysis (GSEA) was used to investigate the possible molecular mechanism of the difference in prognosis between the high-risk group and the low-risk group.

Conclusions

In summary, we identified 13 immune-related lncRNAs signature by constructing a co-expression network that can effectively predict the prognosis of HCC patients, which may become a new prognostic indicator for predicting clinical outcomes and contribute to the clinical decision-making of individual treatment. It is worth further study on the molecular mechanism of hepatocellular carcinoma.

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by the Medical Ethics Committee of Renmin hospital of Guizhou medical university, Guiyang, China.

Consent for Publication

All authors have agreed to publish this manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no conflicts of interests.

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Authors' contributions

Li Liu conceived and designed the experiments. Hao Zhang performed the experiments. She Tian and Zhu Li contributed reagents/materials/analysis tools. Li Liu wrote the paper. All authors read and approved the final manuscript.

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Not applicable.

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Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section.

Table1: Using multivariate Cox regression analysis, 13 immune-related lncRNAs signature was finally identified to predict the prognosis of HCC patients.

Table2: A list of independent prognostic factors associated with HCC patients identified by univariate and multivariate Cox regression analysis.

Table 3: GSEA-KEGG analysis checklist.

Table 4: GSEA-GO analysis checklist.

Supplemental Tables

TableS1: A list of 542 immune-related lncRNAs was identified in hepatocellular carcinoma by constructing a co-expression network.

TableS2: A list of 56 immune-lncRNAs associated with the prognosis of HCC patients was identified by univariate Cox regression analysis.

Figures

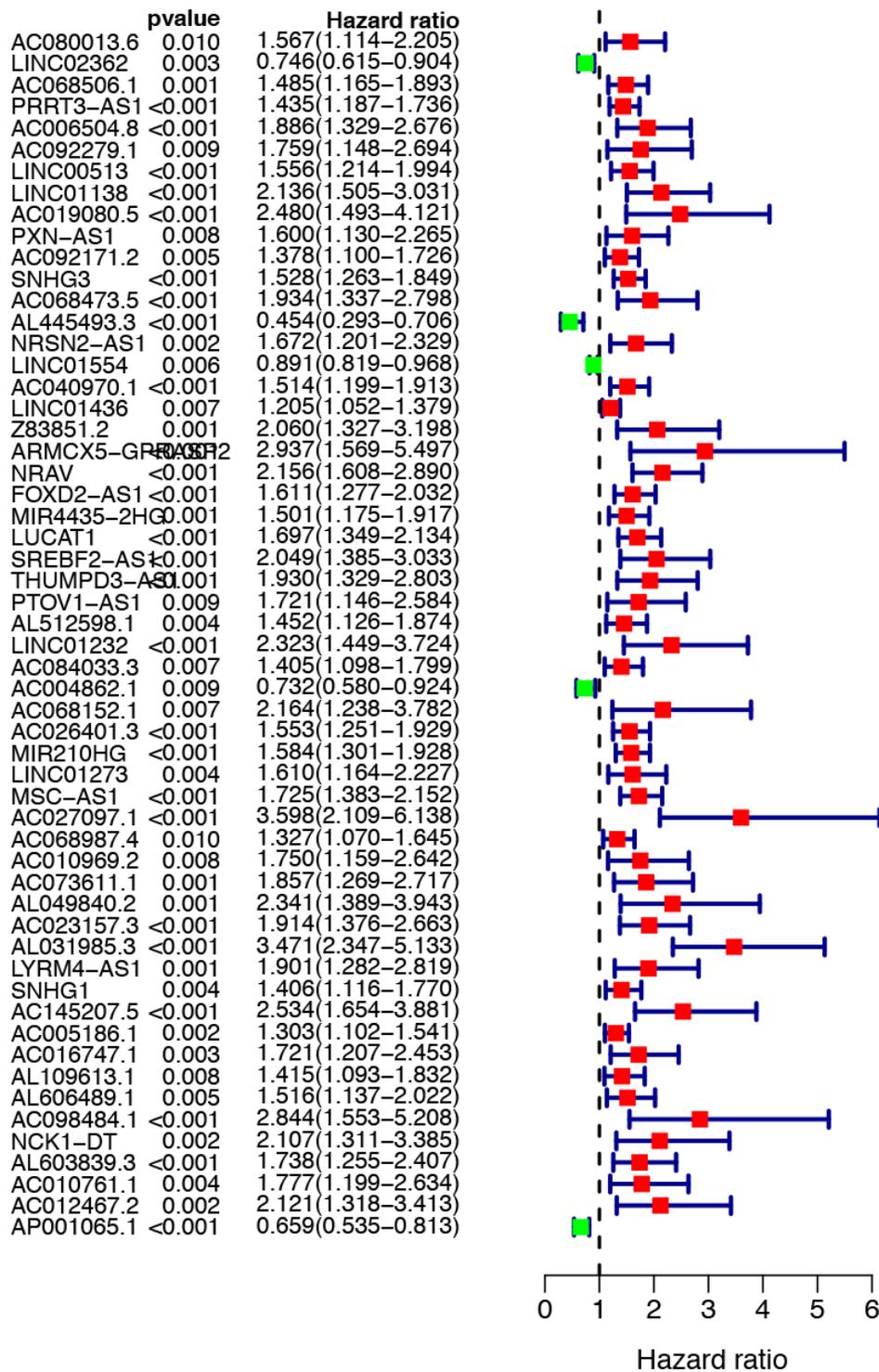


Figure 1

A forest map of 56 prognostic related immune-lncRNAs.

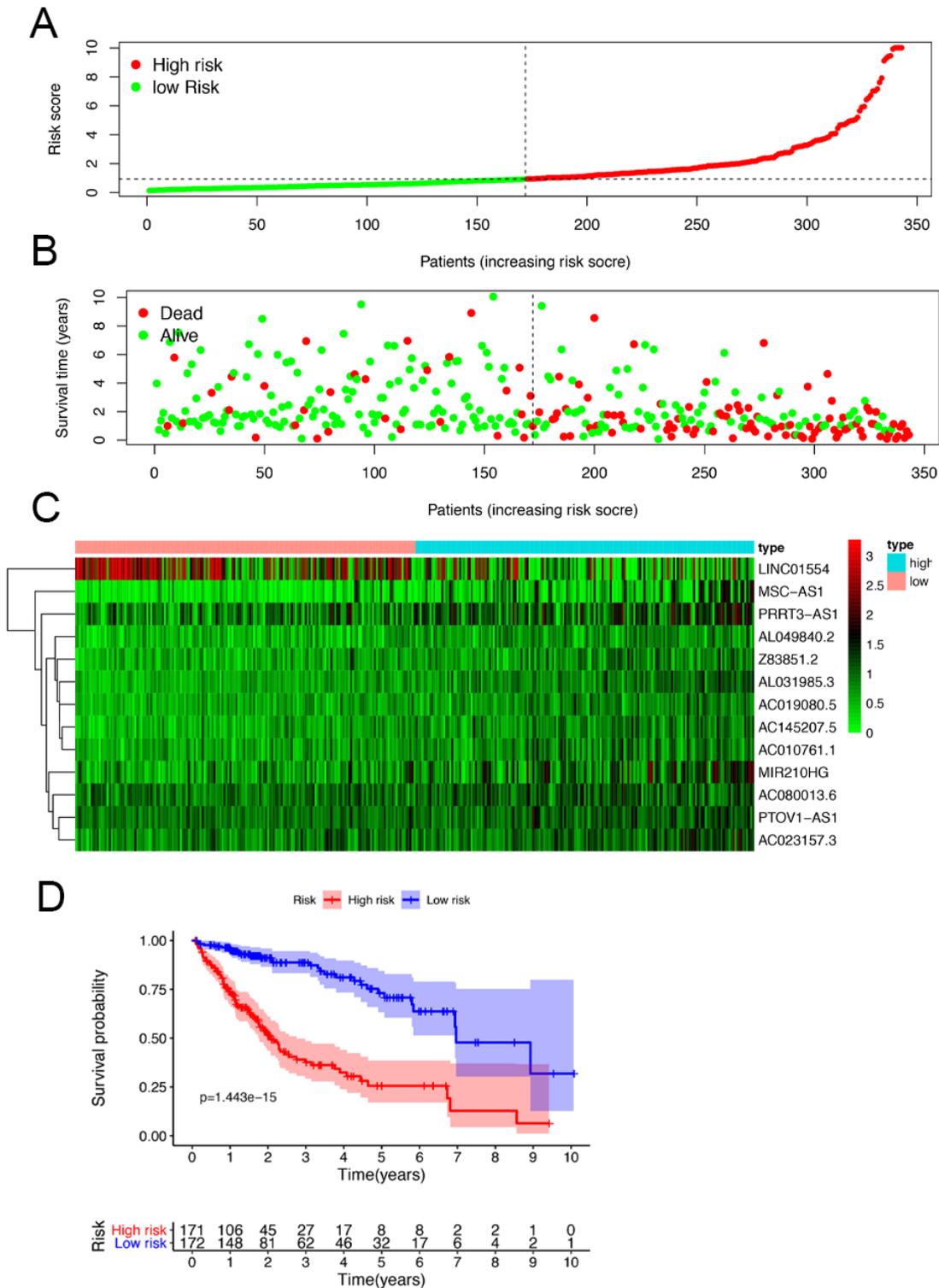


Figure 2

Risk score analysis of 13 immune-lncRNAs signature. (A) HCC patients were divided into two groups according to the median value (high risk group and low risk group). (B) The relationship between risk scores and cancer-related deaths. (C) heat maps of the expression of 13 immune-related lncRNAs identified in the high-risk and low-risk groups. (D) Kaplan-Meier analysis was used to compare the overall

survival (OS) of the high-risk group of HCC patients with the low-risk group, and the overall survival (OS) of the high-risk group was worse.

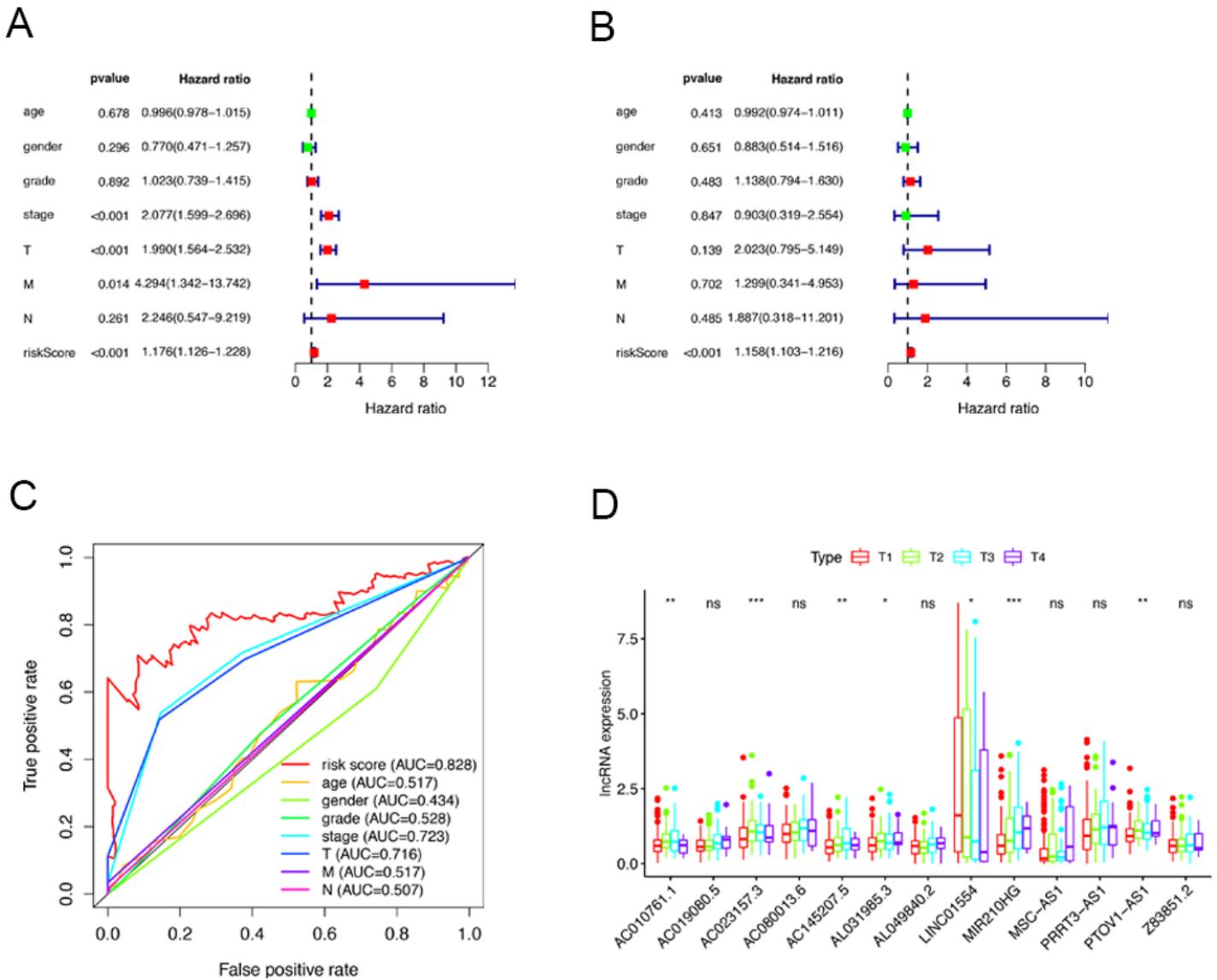


Figure 3

Forest map of independent prognostic factors and the time-dependent receiver operating characteristics (ROC) curve. (A, B) Univariate and multivariate Cox regression analysis was used to identify independent predictors of HCC patients, and $p < 0.001$ was statistically significant. (C) The time-dependent receiver operating characteristics (ROC) curve. (D) Expression of 13 immune-related lncRNAs in T staging.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

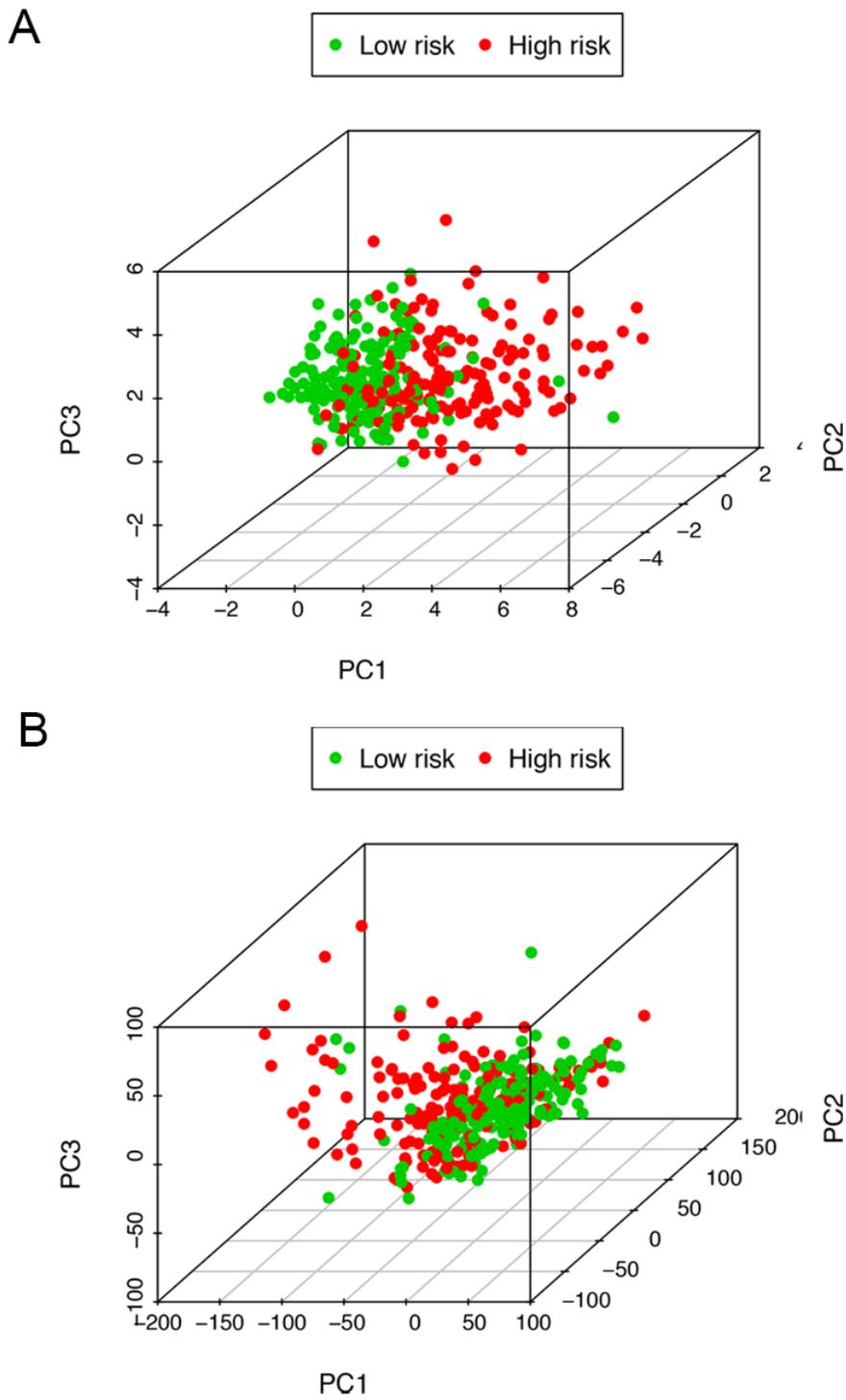


Figure 4

Principal component analysis (PCA). (A) Principal component analysis based on 13 immune-related lncRNAs signature. (B) Principal component analysis based on whole gene expression profile.

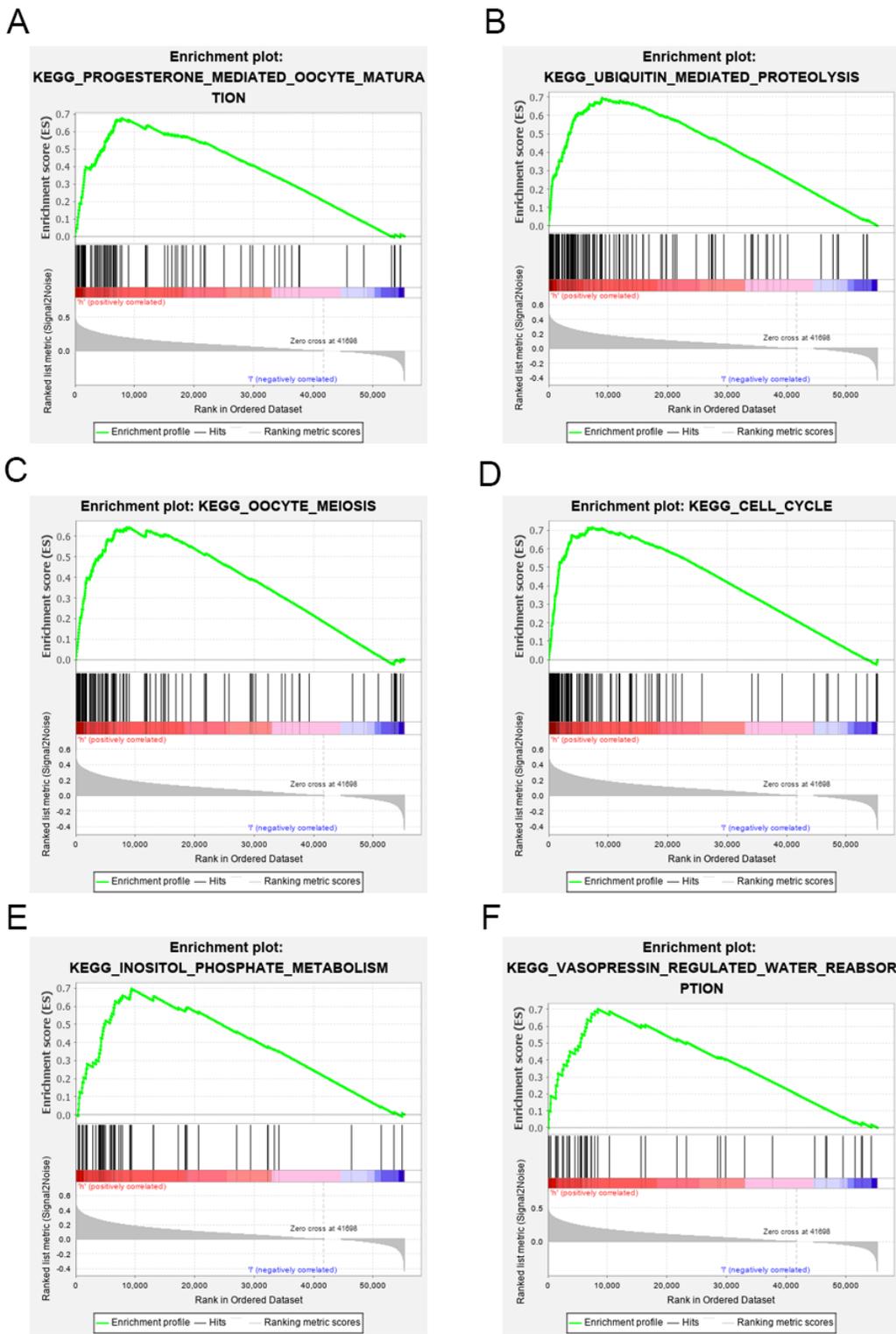


Figure 5

Gene Set Enrichment Analysis (GSEA) of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway indicated significant enrichment of immune phenotype. (A) Progesterone mediated oocyte maturation, (B) Ubiquitin mediated proteolysis, (C) Oocyte meiosis, (D) Cell cycle, (E) Inositol phosphate metabolism, (F) Vasopressin regulated water reabsorption.

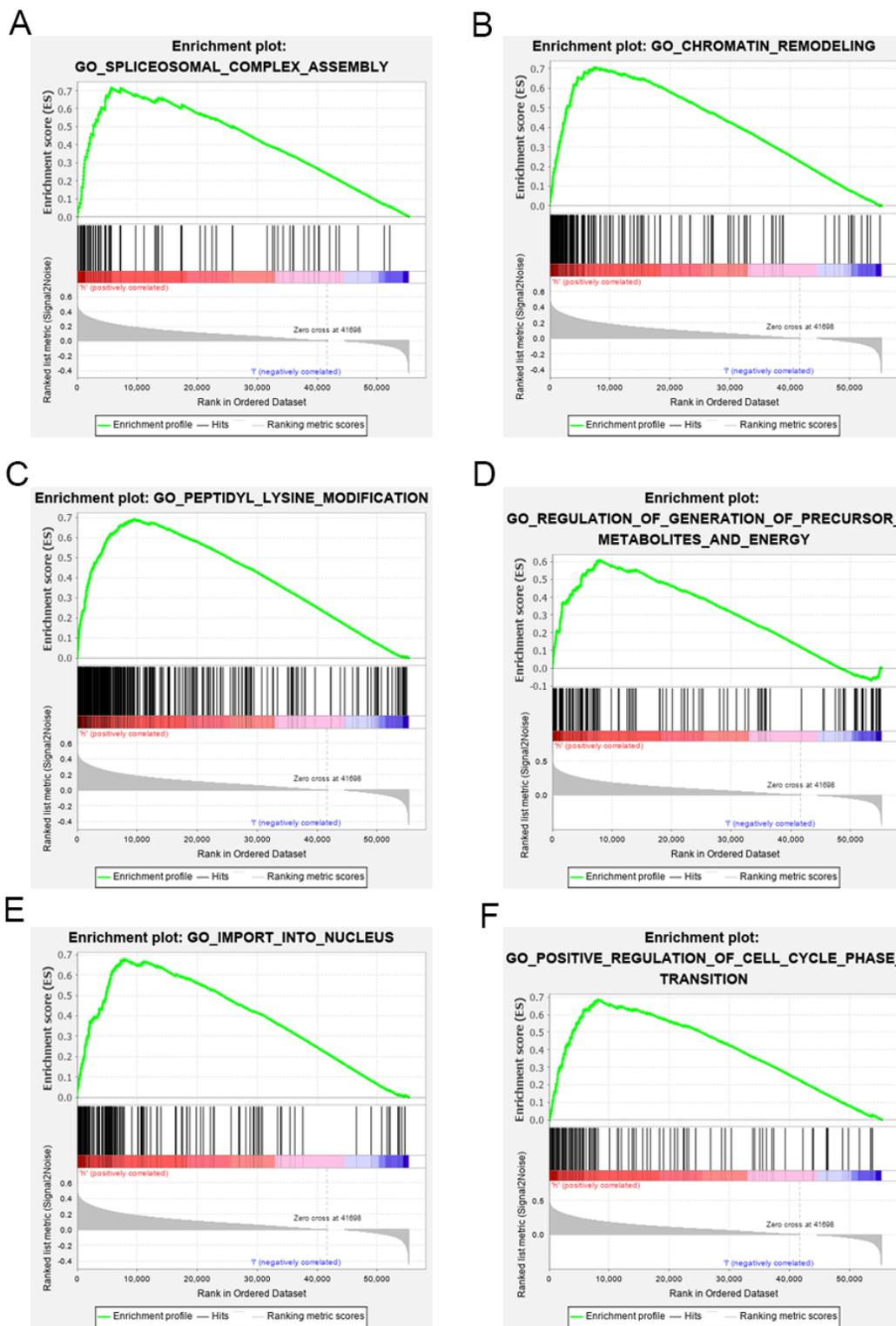


Figure 6

Gene Set Enrichment Analysis (GSEA) of Gene Ontology (GO) indicated significant enrichment of immune phenotype. (A) Spliceosomal complex assembly, (B) Chromatin remodeling, (C) Peptidyl lysine modification, (D) Regulation of generation of precursor metabolites and energy, (E) Import into nucleus, (F) Positive regulation of cell cycle phase transition.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table.S1.xlsx](#)
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- [Table4.xlsx](#)
- [TableS2.xlsx](#)
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- [Table2.xlsx](#)