Kidney Renal Clear Cell Carcinoma: Development and Validation of Prognostic Index of Necroptosis-Related Genes

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Primary research

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Abstract

**Background:** Renal carcinoma is a frequent kind of malignant tumour of the urinary system, and its prevalence is increasing.

**Methods:** The predictive significance of Necroptosis-related genes (NRGs) in 539 Kidney renal clear cell carcinoma (KIRC) samples from The Cancer Genome Atlas (TCGA) datasets was investigated. We wanted to show how NRGs interact with immunological checkpoints and m6A in KIRC. In the Kyoto Encyclopedia of Genes and Genomes, gene set enrichment analysis was employed to study gene expression enrichment. Lasso regression was used to build the predictive model. According to a co-expression study, gene expression is directly related to necroptosis. In the absence of additional clinical signs, NRGs was shown to be partly overexpressed in high-risk individuals, indicating that they might be used in a model to predict KIRC prognosis. GSEA identified immunological and tumour-related pathways in the high-risk group.

**Results:** According to TCGA, immune checkpoint and m6A genes differ considerably between the low-risk and high-risk groups. PLA2G4D, H2AC17, H2AC7, IRF9, and other genes varied between the two risk groups. NRGs are linked to the onset and development of KIRC.

**Conclusions:** Using matching predictive models, the prognosis of KIRC patients may be predicted. NRGs and the association of immunological checkpoints and m6A with NRGs in KIRC may represent potential therapeutic targets that should be investigated further.

1 Introduction

Renal carcinoma is a frequent kind of malignant tumour of the urinary system, and its prevalence is increasing. Kidney renal clear cell carcinoma (KIRC) is the most prevalent renal parenchymal carcinoma, accounting for around 80–86% of all cases. Early KIRC is often asymptomatic or only manifests as systemic symptoms such as fever and tiredness. Early clinical diagnosis is challenging due to a lack of suitable screening methods, and most patients are discovered as the tumour volume grows. The preferred therapy for kidney cancer is now radical nephrectomy. Although KIRC is the least malignant of kidney tumours, it has a high incidence of metastasis by blood transport, with around 60% of patients having the chance of metastasis. Patients with metastatic KIRC have an abysmal prognosis, are typically resistant to chemotherapy and radiation, have few effective therapies, and have a 5-year survival rate of fewer than 10%. Given the limits of KIRC therapy, there is an urgent need for accurate new predictive models, and research into genes closely linked to KIRC is likely to give new techniques. The theoretical basis for novel tumour gene-targeted therapy, making targeted therapy more practical.

Resistance to apoptosis is a significant barrier that causes chemotherapy to fail during cancer treatment. It is thought that avoiding the apoptotic pathway to enhance cancer cell death is a viable solution to this problem. Necroptosis is a caspase-independent, controlled necrotic cell death process mediated
predominantly by receptor-interacting Protein 1 (RIP1), RIP3, and Mixed Lineage Kinase Domain-Like Protein (MLKL). It plays a variety of roles in cancer. The expression of essential regulators of the necroptotic pathway is typically downregulated in cancer cells, indicating that cancer cells may be able to avoid necroptosis to live. Nonetheless, essential mediators’ expression is enhanced in certain forms of cancer. Although necroptosis can elicit significant adaptive immune responses that protect against tumour growth, the recruited inflammatory response can promote carcinogenesis and cancer spread, and necroptosis can create an immunosuppressive tumour microenvironment. Despite evidence suggesting that necroptosis has an antimetastatic impact on cancer, it is thought to enhance oncogenesis and cancer spread. Despite this, there have been only a few sequence-based studies on abnormal gene expression and its link with overall survival (OS) in KIRC patients with Necroptosis.

Immune checkpoint-related gene profiles in KIRC patients could assist detect treatment responsiveness, assess risks, and predict survival. Even though there has been little research on the association between NRGs and KIRC, it is crucial to investigate the interaction of NRGs, checkpoints, and m6a with KIRC clinicopathological tumour characteristics. The reason and mechanism of KIRC's aberrant gene expression and necroptosis are unknown at this time. Transcriptional maps of NRGs modification in KIRC patients are required to understand the NRGs pathways that influence the prognosis of KIRC patients. To accurately assess and predict overall survival in KIRC patients, immune checkpoint-related gene profiles can be used to predict therapy responsiveness. Understanding how NRGs affect KIRC progression may lead to discovering a biomarker that can be used as a therapeutic target.

This work aimed to identify NRGs whose expression is connected with KIRC patient prognosis to build a predictive model for KIRC prognosis prediction. We can help create innovative KIRC therapeutic targets and pharmaceutical methods by better understanding the invasion of NRGs and their relevant targets, pathways, immune checkpoints, and m6a.

## 2 Materials And Methods

### 2.1 Datasets and NRGs

We obtained KIRC gene expression patterns and clinical data from The Cancer Genome Atlas (TCGA) using the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/). The strategy is to look at gene expression profiles (Cases: kidney and TCGA and TCGA-KIRC; Files: transcriptome profiling and Gene Expression Quantification and HTSeq-FPKM). Clinical research information (Cases: kidney and TCGA and TCGA-KIRC; Files: clinical and bcr xml). On October 14, 2021, the expression patterns of 539 instances of KIRC and 72 cases of normal tissues were included in the TCGA common database. The clinical characteristics of the patients are summarized in Table 1. Furthermore, we discovered 159 NRGs in total (Table S1).
Table 1
The clinical characteristics of patients in the TCGA dataset.

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2.2 Annotation of genes

The Genome Reference Consortium Human Build 38 (GRCh38) lncRNA annotation file was acquired from the GENCODE website to annotate the lncRNAs in the TCGA dataset. The transcriptome data and human configuration files were matched and sorted using Perl software (https://www.perl.org/), and the relevant mRNA and lncRNA gene expression data were acquired. Using information from the ensemble database (http://asia.ensembl.org/info/data/index.html), the gene IDs were converted to gene names. The R4.1.0 Limma program was used to extract NRGs expression data based on the gene expression matrix of previously obtained NRGs expression profile data.

2.3 Identification of Necroptosi-related DEGs and analysis of mutation rates of DEGs

Pearson correlation was used to investigate the link between NRGs and KIRC. The Limma package’s correlation test was used to analyze the expression of NRGs after eliminating the standard samples and using p<0.05 and corFilter=0.7 as screening criteria. Gender, age, Grade, stage, T, M, N, survival status, and survival duration were among the clinical-pathological data obtained from KIRC patients. FDR<0.05 and |log2FC|≥1 were used to evaluate a significant change in NRG expression. First, we looked into the
function of necroptosis-related differentially expressed genes that were both up and downregulated (DEGs). We investigated the genetic changes of these genes because of the significant clinical consequences of these NRGs. DEG mutation rates were examined using Cbioportal (http://www.cbioportal.org/). Kidney renal clear cell carcinoma strategy (TCGA, Firehose Legacy).

### 2.4 Functional enrichment of the differentially expressed NRGs

Using Gene Ontology, the biological pathways associated with the DEGs were then examined (GO). Biological processes (BP), molecular functions (MF), and cellular components (CC) controlled by the differentially expressed NRGs were further investigated using R software, clusterProfiler, org.Hs.eg.db, enrichplot, and ggplot2 package based on KEGG data.

### 2.5 Development of NRGs prognostic signature

To build a prognostic model, NRGs signature was constructed using Lasso-penalized Cox regression and Univariate Cox regression analysis, stratified by risk score (Coefficient DEGs$_1$ × expression of DEGs$_1$) + (Coefficient DEGs$_2$ × expression of DEGs$_2$) +... + (Coefficient DEGs$_n$ × expression DEGs$_n$). Each KIRC patient’s associated risk score was further evaluated. Based on the median score, the DEGs were divided into three subgroups: low-risk (< median number) and high-risk (≥ median number). The low-risk (50%) and high-risk (50%) groups were identified in Lasso regression, and the appropriate plots were generated. Following visualization, the confidence interval and risk ratio were computed, and the forest diagram was created. The survival curves for the high-risk and low-risk groups were formed and compared. To test the accuracy of our model for predicting survival in KIRC, we used the timeROC program to create a similar receiver-operating characteristics (ROC) curve. The risk and survival status of NRGs were studied concerning the risk curve formed by the risk score. An independent prognosis study was carried out to ensure that our model was unaffected by other clinical prognostic variables influencing the patients’ outcomes. To determine hazard ratios, the researchers employed multivariate and univariate models. To ascertain the relationship between clinical variables and our risk prediction model and differentiate between high-risk and low-risk NRGs cases. Analyses of risk and clinical association have been conducted. The Heatmap was created using the Heatmap and limma packages. Decision Curve Analysis (DCA) was built to illustrate the validity of our model further.

### 2.6 GSEA enrichment analyses and the predictive nomogram

GSEA (https://www.gsea-msigdb.org/gsea/index.jsp) was used to find differences in linked functions and pathways in several samples, and data was imported using the PERL programming language. The associated score and graphs were used to determine whether or not the functions and routes in the various Risk categories were dynamic (c2.cp.kegg.v.7.2.symbols.gmt,Risk.cls#h versus l). Each sample was classified as ‘H’ or ‘L’ depending on whether there was a high-risk cluster of prognosis-related NRGs. The number of permutations, no collapse, and phenotype was all set to 1000, no collapse, and
phenotypic. The gene list was sorted in real mode, with the genes listed in decreasing order. To rank the genes, the 'Signal2Noise' measure was used. The 'meandiv' normalization method was used, and the difference was statistically significant with an FDR<0.05. A nomogram was created by combining the predictive signals to predict the 1, 2, and 3-year OS of KIRC patients. Furthermore, because these NRGs have significant clinical consequences, we looked into the relationship between NRGs, checkpoints, and m6a.

3 Results

We identified 44 necroptosis-related DEGs and 12 risk NRGs based on expression differences between tumour and normal tissues. GSEA was used to uncover latent signalling pathways implicated in the development and progression of KIRC, and lasso regression was used to build a suitable prediction model.

3.1 Differentially expressed NRGs

Compared to normal samples, we identified 44 DEGs associated with necroptosis (8 downregulated and 36 upregulated; Table S2) (Figure.2a). The univariate COX research identified 22 significant NRGs, which were then incorporated in the multivariate COX analysis. In all, 22 distinct NRGs (TRAF2, RBCK1, SLC25A4, SLC25A5, MAPK10, JMJD7PLA2G4B, PLA2G4B, PLA2G4D, PYCARD, TNFRSF10B, FASLG, IFNG, IFNAR2, IFNGR2, JAK3, STAT4, IRF9, TLR3, ZBP1, BID, H2AC17, H2AC7) were identified to be (Table S4). As a result, we computed risk ratings for the NRGs and created a prognostic signature. We explored their genetic alterations because these NRGs have significant clinical effects and observed that truncating and missense mutations were the two most common types of mutations (Figure.2c). A total of 6 genes showed a 1% mutation rate, with CAMK2A being the most often modified (14%).

3.2 Enrichment Analysis of necroptosis-related genes

526 core targets were discovered by GO enrichment analysis, including MF, CC, and BP. The MF mainly involves signaling receptor activator activity (GO:0030546), receptor ligand activity (GO:0048018), ubiquitin-like protein transferase activity (GO:0019787), phospholipid binding (GO:0005543). The CC mainly involves membrane raft (GO:0045121), membrane microdomain (GO:0098857), protein-DNA complex (GO:0032993), DNA packaging complex (GO:0044815). The BP mainly involves positive regulation of anion transport (GO:1903793), signal release (GO:0023061), T cell activation (GO:0042110), regulation of immune effector process (GO:0002697). In addition, the main signaling pathways were identified by KEGG enrichment analysis, revealed the over-expressed genes were mainly involved in Herpes simplex virus 1 infection (hsa05168), Pathways of neurodegeneration-multiple diseases (hsa05022), Alzheimer disease (hsa05010), PI3K-Akt signaling pathway (hsa04151), Huntington disease (hsa05016), Cytokine-cytokine receptor interaction (hsa04060), MAPK signaling pathway (hsa04010) (Figure.3 and Table S3).

3.3 Survival results and multivariate examination
A Kaplan-Meier analysis showed that high-risk NRGs signatures were associated with a shorter survival time (P<0.001. Figure 4a). Meanwhile, the AUC for NRG signature was 0.769, indicating that it outperformed standard clinicopathological characteristics in predicting KIRC prognosis (Figure 4b-c). We observed that the patient's risk score was inversely related to the survival of KIRC patients using a risk survival status plot. Surprisingly, most of the new NRGs discovered in this study showed a negative relationship with our risk model, indicating that more research is needed (Figure 4d). For 1, 2, and 3-year survival rates, the AUC predictive value of the unique NRGs signature was 0.769, 0.737, and 0.736, respectively (Figure 4e). NRGs, PLA2G4D, H2AC17, H2AC7, IRF9, IFNG, FASLG, STAT4, TLR3, JAK3, IFNAR2, and BID were all substantially represented in a high-risk group, indicating that all of them may be detrimental to KIRC patients' prognosis (Figure 4f). COX analysis revealed that the NRGs signature (HR: 1.195, 95 CI: 1.113-1.282) and Age (HR: 1.032, 95 CI: 1.011-1.052) were the most important independent predictors of KIRC patients' survival (Figure 5a-b). Figure 5c demonstrates the connection between Necroptosis and RNA. The hybrid nomogram (Figure 6) that integrated clinicopathological features and the NRGs prognostic signal was stable and reliable, and therefore may be employed in the therapy of KIRC patients.

3.4 The necroptosis-related signature is an independent prognostic factor for KIRC patients

Clinicopathological studies were carried out to examine the connections between clinical markers and the risk profile (Figures 7a–d). The signature was associated with tumor stage (p=1.875e-08), T stage (p=1.977e-10), M stage (p=2.871e-06), and N stage (p=1.348e-08). The survival rates differed significantly between the high-risk and low-risk groups. Patients in the high-risk group had shorter overall survival (OS) than those in the low-risk group. Using OS ROC curves, the prediction performance of the NRGs risk signature was stated (Figure 7e). Thus, by combining university and multivariate Cox-regression analysis data, we discovered that the necroptosis-related signature might be used as an independent predictor in clinical practice. Furthermore, we created a heatmap of clinical characteristics for the NRGs. We discovered that patients' Gender, Grade, Stage, T, M, and N were distributed differently across the low- and high-risk groupings (Figure 7f).

3.5 Gene set enrichment analyses

The majority of NRGs prognostic signature regulated immunological and tumor-related pathways such as homologous recombination, ribosome, primary immunodeficiency, intestinal immune network for iga synthesis, proteasome, p53 signaling pathway, and so on, according to gene set enrichment analysis (GSEA). Figure 8 depicts the top six enriched functions or pathways for each cluster (Table S5). Both the FDR q-value and the FWER p-value were <0.05. As a result, the 'p53 signaling pathway' was shown to be the most enriched, and some of the genes were found to be positively associated with H or L.

3.6 Analysis of the correlation between NRGs with immune checkpoints and m6A
Given the significance of checkpoint inhibitor-based immunotherapies, we investigated differences in immune checkpoint expression between the two groups. We discovered a significant difference in the face of CD44, TNFRSF8, CD27, TMIGD2, HHLA2, LGALS9, and other genes between the two patient groups (Figure 9a). When the expression of NRGs was compared between the high and low-risk groups, YTHDC1, YTHDC2, YTHDF2, ZC3H13, FTOALKBH5, METTL14, METTL3, and RBM15 were shown to be significant (Figure 9b).

4 Discussion

Because of its advanced stages and debilitating disease, treating KIRC is a severe clinical concern. At all times, the molecular discovery of diagnostic biomarkers and therapeutic targets for KIRC should be prioritized. According to previous investigations, necroptosis is involved in pathological cell death related to acute renal injury in the kidney research field. It is an alternative mode of programmed cell death that overcomes apoptosis resistance, and it has the potential to activate and boost antitumor immunity in cancer therapy. Necroptosis has the potential to act as a tumour suppressor, making it a viable cancer treatment option. However, it is unknown how it impacts KIRC development via regulating NRGs. This researcher evaluated the role of critical targets, pathways, and immune checkpoint inhibitors in the prognosis of KIRC. This study's findings suggested a viable biomarker and treatment target.

We gathered NRGs expression data in this study and differentiated between mRNA and IncRNA. The connection between NRGs expression and RNAs were investigated using co-expression analysis. Using the co-expression network diagram, we discovered that many RNAs in KIRC were linked to NRGs. Following that, we identified 44 DEGs related to necroptosis. KEGG analysis found that the genes were primarily involved in necroptosis, apoptosis, PI3K-Akt signaling pathway, MAPK signaling pathway, and p53 signaling pathway. A growing body of data shows that apoptosis and autophagy may also play essential roles in the genesis and progression of diabetic kidney disease; they are the Four Horsemen of the Apocalypse. By activating the RIP1-RIP3-MLKL signaling pathway, PI3K promotes tumour necrosis factor-induced necroptosis. By activating p38 MAPK, tumour suppressor death-associated protein kinase 1 inhibits necroptosis. Visibility. Necroptosis plays a key role in KIRC.

The NRGs were split into two groups to examine their potential roles in KIRC: high-risk and low-risk. The confidence interval and hazard ratio were computed using data on prognosis-related genes. NRGs were shown to be significantly related to the KIRC prognosis in a university Cox regression study. This study discovered 12 NRGs that have been linked to prognosis and exhibited different expressions in high-risk and low-risk individuals. Some NRGs were found to be overexpressed in high-risk individuals, whereas others were overexpressed in low-risk individuals (P<0.05). We looked examined the involvement of NRGs in KIRC further. A survival study based on gene subtypes was used to determine the predictive value of NRGs. Patients with low-risk NRGs outperformed those with high-risk NRGs. CAMK2A, PYGM, BIRC3, and H2AW were highly expressed in the high-risk group, indicating that they may be KIRC oncology genes, according to the NRGs risk score. Furthermore, the previously stated NRGs may be used as a therapeutic
target for KIRC. NRGs were also connected to patient outcomes in the KIRC study. Only a tiny amount of research has been done on gene alterations linked to necroptosis. More study is required to fully comprehend the process of NRG modification and identification and confirm our findings.

In addition, we studied and estimated the connection between NRGs, immunological checkpoints, and m\(^6\)a. In ICI-resistant malignancies, de novo necroptosis can generate an inflammatory milieu that promotes tumour sensitivity to immune checkpoint inhibitors (ICI), increasing necroptosis and reducing tumour development in autochthonous tumours\(^6\). MIR155HG is a prognostic biomarker that has been linked to immune infiltration and the expression of immune checkpoint molecules in a variety of malignancies, including KIRC\(^7\). The relationship between SLC41A3 expression and immune cell infiltration, also known as an immunological checkpoint\(^8\). Only a tiny amount of research has been conducted on the relationship between ICI, m\(^6\)a, and necroptosis. Even though a bit of study has been shown on NRGs and KIRC, based on the findings provided above, we may conclude that a shift in NRGs is associated with the development and progression of KIRC.

In GSEA, the p53 signaling pathway was found to be the most significantly enriched pathway. Recent research has revealed that p53 can not only produce necrosis via opening the mitochondrial permeability transition pore, but it can also directly interact with cyclophilin D (CypD) to open the permeability transition pore (PTP) in the oxidative stress response, thereby contributing to necrosis\(^9\). The development and progression of renal cell carcinoma are strongly linked to ubiquitin-like modified activating enzyme 2 (UBA2). UBA2 suppresses apoptosis in renal cell carcinoma by inhibiting the p53 pathway and increasing renal cell carcinoma incidence and progression\(^10\). Taking the features mentioned above into account, NRGs may impact KIRC cell migration and proliferation via altering the P53 SIGNALING PATHWAY. According to the NRGs predictive model, the low-risk subtype had a higher survival rate than the high-risk subtype. Furthermore, our model predicts KIRC patient survival with excellent accuracy. A rise in the risk score is linked to an increase in mortality rates and the high-risk ratio. Other clinical prognostic factors that may influence patient outcomes were unaffected by our approach. The idea might be applied to a wide range of clinical conditions. Based on our findings and data from the literature, NRGs appear to be valid biomarkers for predicting KIRC patient outcomes.

Although our research provides some theoretical underpinnings and research recommendations, it has limits. To begin, we built and validated an NRGs prediction signature using the TCGA dataset. We could not find sufficient external data from other publicly available sources to assess the model’s reliability. Second, we focused our preliminary expression research on the signature’s 12 risk-NRGs. Despite this, no more functional or mechanistic study was carried out. Finally, no investigations were conducted in KIRC to verify the connection between prognostic genes and necroptosis. However, to fully comprehend the facts indicated above, we will undertake an additional inquiry.

### 5 Conclusions
In conclusion, we looked for prognosis-related NRGs in the TCGA database by examining the expression patterns and clinical data of KIRC samples. In 539 KIRC patients, 12 anticipated NRGs were identified as part of the necroptosis regulation. For KIRC, it has a substantial predictive value. Our findings contribute to our knowledge of the relationship between ICI, m⁶a, and necroptosis, potentially paving the way for new therapeutic targets and prognostic markers. It is desirable since our findings will aid in identifying NRGs that promote KIRC growth, allowing us to understand more about their possible involvement in the genesis and progression of KIRC malignancies.

**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>KIRC</td>
<td>Kidney renal clear cell carcinoma</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
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<td>GO</td>
<td>Gene Ontology</td>
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<td>NRGs</td>
<td>Necroptosis-related genes</td>
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<td>AUC</td>
<td>areas under the curve</td>
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<td>BP</td>
<td>Biological processes</td>
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<td>CC</td>
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<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
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**Declarations**

**Data availability**

Patients who granted informed consent to use their data have been included in the public-accessible TCGA database. At their leisure, users can get and publish relevant articles depending on the needed data. Our study has no ethical problems or conflicts of interest because it is based on open-source data.

**Ethics approval and consent to participation**

Because this is not a clinical experiment, ethical approval and agreement to participate are not required.

**Consent for publication**

All authors have reviewed and approved this article for publication consideration.

**Competing interests**

The authors declare no competing financial interests.

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Fund


Author Contributions

The manuscript was written and corrected by Zixuan Wu. Xuyan Huang and Minjie Cai oversee data gathering. Peidong Huang planned and designed this essay, and he was in charge of modifying the syntax and revising the writing. Zunhui Guan corrected the manuscript. The final manuscript version has been read and approved by all of the writers.

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**Figures**
Figure 1

Framework based on an integration strategy of NRGs.
Figure 2

The differentially expressed NRGs. a: The boxplot of the differentially expressed NRGs. b: Forest plot of significant NRGs. c: Mutations in NRGs. (N: normal; T: tumor).
Figure 3

GO and KEGG analyses for necroptosis-related differentially expressed genes. (a + b) GO (c + d) KEGG.

Figure 4

GO and KEGG analyses for necroptosis-related differentially expressed genes. (a + b) GO (c + d) KEGG.
NRGs signature. (a) Kaplan-Meier curves result, (b). The AUC values of the risk factors, (c). The DCA of the risk factors. (d). Risk survival status plot, (e). The AUC of the for the prediction of 1, 2, 3-year survival rate of KIRC, (f). Heatmap of different NRGs.

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</table>

**Figure 5**

COX analysis for the expression of NRGs, both univariate and multivariate. (a). univariate, (b). multivariate, (c). The relationship between NRGs and RNA expression.
Figure 6

A nomogram for prognostic NRGs as well as clinic-pathological variables.
Figure 7

The necroptosis-related signature in the cohorts. a: Grade. b: stage. c: T. d: M. e: N. f: The ROC analysis of OS for the signature and the clinicopathologic parameters. g: Heatmap.
Figure 8

Gene set enrichment analyses for NRGs.
Figure 9

(a). Immune checkpoint expression in high and low KIRC risk groups. (b). The expression of NRGs in KIRC risk groups with high and low KIRC risk.

Supplementary Files

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- appendix.doc