Comprehensive Analysis of CXCL genes in Human Kidney Clear Cell Carcinoma

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Abstract

Introduction: the predictive value for survival outcomes and mechanisms of the CXCL gene family in kidney Clear Cell Carcinoma (KIRC).

Objective: We evaluated the predictive value for survival outcomes and mechanisms of the CXCL gene family in Kidney Clear Cell Carcinoma (KIRC).

Methods: Transcriptional and survival data of CXCL genes in KIRC patients were retrieved from Oncomine and GEPIA databases to assess their functions. Mutations' data were downloaded from the cBioPortal, while immune cell components data were obtained from TIMER databases. Enrichment analysis was performed using the STRING database. In vitro assays were performed to validate our findings.

Results: A total of 8 CXCL (CXCL2/5/9/10/11/12/13/16) genes were found to be differentially expressed in KIRC tissues. Among them, only CXCL2 and CXCL5 had survival values. In vitro assays revealed that upregulated CXCL2 promotes renal cancer cell proliferation, migration and invasion. Additionally, CXCL2 exhibited an effect on three KIRC immune cell components (B cells, CD4 T cells, and Neutrophils). After evaluating the miRNA sequences upstream of CXCL2, it was established that hsa-miR-532-5p/CXCL2 pathways are potentially involved in the regulatory mechanisms. Finally, we established a prospective signature (CXCL1/5/13) for improved survival outcomes among KIRC patients.

Conclusion: Among KIRC patients, 8 CXCL genes were differentially expressed, however, only CXCL2 and CXCL5 exhibited a significant survival value. Upregulated CXCL2 promotes renal cancer cell progression.

Introduction

Kidney Clear Cell Carcinoma (KIRC) is the most common type of kidney cancer. Various risk factors, including HIF2-vegf axis and other inflammatory mediators, are associated with KIRC initiation and progression(1). Despite significant advances in diagnosis, treatment, and prognosis, clinical outcomes for KIRC patients are unsatisfactory.

The CXCL gene family is a group of CXCL genes that secrete proteins involved in immune-regulatory and inflammatory processes(2). These genes may be involved in tumor infiltration and immune cell components(3). Studies have evaluated the prognostic value for certain CXCL proteins in KIRC patients. Upregulated CXCL8 recruit tumor-associated neutrophils to promote renal cell carcinoma progression(4). However, the prognostic value for the whole CXCL gene family among KIRC patients has not been little researched.

In this study, we investigated the expressions of CXCL genes, the correlations between CXCL genes and clinical information, mutation status, and miRNA regulation of CXCL genes, immune cell components in KIRC patients. The aim was to identify novel survival and prognostic factors for KIRC patients. Moreover, CXCL2 levels and associated functions were evaluated in a human renal clear cell carcinoma cell line.

Materials And Methods

Target gene expression and clinical information

The CXCL genes were searched in the GeneBank (https://www.ncbi.nlm.nih.gov/gene/) website and a list of CXCL genes in the human population retrieved(5). Gene expression RNAseq data and clinical phenotype of TCGA kidney Clear Cell Carcinoma (KIRC) were downloaded from the UCSC Xena database (https://xenabrowser.net/datapages/)(6).

Differential gene expression analysis

The oncomine database (https://www.oncomine.org/resource/main.html) is a cancer microarray database and an integrated data-mining platform(7). Oncomine was used to assess CXCL genes in various cancer types. GEPIA (http://gepia.cancer-pku.cn/), a web-based tool for expression and functional analyses of TCGA and GTEx data, is important for differential expression analysis, profile plotting, correlation analysis, survival analysis, among other analyses(8). In this study, GEPIA was used to determine the expressions of CXCL genes, as well as correlations between expression levels and pathological stages for several cancer types.

Construction of the protein-protein interaction (PPI) network and functional enrichment analysis

The top 100 CXCL correlated genes were obtained from the STRING database (https://string-db.org/)(9). The functions of the intersection were used to identify common correlated genes for all target CXCLs genes. We established the PPI network and performed functional enrichment analyses using the STRING database. Results from the STRING database were downloaded and visualized using the Cytoscape software(10).

Survival analysis

We used GEPIA to perform Kaplan Meier (KM) analysis on KIRC target genes. “Survival” and “glmnt” packages in R were used to compute time-dependent ROC and nomogram models(11). To evaluate clinical information and target genes for survival value, we employed univariate Cox proportional hazard regression(12).

Mutation analysis

The cBioPortal online tool (https://www.cbioportal.org/) is a web resource for exploring, visualizing, and analyzing multi-dimensional cancer genomics data(13). We used cBioPortal to analyze the mutations of

target CXCL genes in KIRC patients. UCSC Xena database (http://xena.ucsc.edu/) is a genome-related database comprised of over 200 public databases. It is used to analyze copy number variations, methylation status, somatic mutations, gene as well as protein expressions. In this study, the UCSC Xena analysis function was used to assess target CXCL copy number variations, methylation status, and somatic mutations.

Upstream miRNA prediction and analysis

To investigate upstream miRNAs of target CXCL genes, we used four miRNA prediction databases (miRDB, miRWalk, RNA22, and TargetScan)(14–17). Correlations between miRNAs and CXCL genes was assessed using the starBase database (http://starbase.sysu.edu.cn/)(18). The starBase database was used to assess the survival value and expression levels of miRNAs in KIRC patients.

Analysis of tumor-infiltrating immune cells

TIMER (http://timer.cistrome.org/) is a web server for comprehensive analysis of tumor-infiltrating immune cells(19). Six tumor-infiltrating cells (B cells, CD4 + T cells, CD8 + T cells, neutrophils, macrophages, and dendritic cells) were evaluated using TIMER. Correlations between CXCL gene targets and molecular markers for various immune cells were assessed.

Assessment of the correlation between risk scores and clinical information

Univariate Cox proportional hazard regression was used to analyze the association between RNAseq expression and survival data for all CXCL genes in the KIRC dataset. Multivariate Cox regression analysis was performed to identify hub survival genes. “Survival” and “glmnt” R packages were used for the above calculations, and $p < 0.05$ was set as the threshold for statistical significance.

The risk score was determined using multivariate Cox regression analysis. Moreover, we evaluated the clinical features, tumor-infiltrating immune cells, and responses to immune therapy in KIRC patients with varying risk scores.

Cell lines

The 293T cell line (a human embryonic kidney cell line), and caki-1 cell line (a human renal clear cell carcinoma cell line) were acquired from the Chinese national collection of authenticated cell cultures. These cell lines were used to assess CXCL levels between benign and malignant renal cells. Cell cultures were performed based on the guidelines of the Chinese national collection of authenticated cell cultures.

Quantitative real-time polymerase chain reaction (Q-PCR) and western blot (WB) analyses

These analyses were performed to analyze CXCL gene expression levels between benign and malignant renal cells. Both assays were performed as previously described. Q-PCR was performed using the TOYOBO ReverTra Ace Q-PCR RT system with TOYOBO PCR primers(20). In WB analysis, primary antibodies against GAPDH and CXCL genes as well as secondary antibodies were purchased from Boiss (Beijing, china)(21). GAPDH was the internal reference. Western blot images were analyzed using the Image J software.

Cell transfection

CXCL2 siRNAs were synthesized by the General biology company (Xuzhou, China)(22). We designed three CXCL2 siRNAs and performed Q-PCR to compare their cell transfection efficiencies. The lowest expression of CXCL2 siRNA would be picked to do the function experiment.

CCK-8 and Transwell assays

The CCK-8 assay, which was used to evaluate renal carcinoma cell proliferation, was performed using the Enhanced Cell Counting Kit-8 (Dojindo, Japan), as instructed by the manufacturer(23). Transwell assays were performed to assess the migratory and invasive capacities of renal carcinoma cells(24).

Results

Target genes in KIRC exhibit varied expressions between cancer and normal tissues.

The CXCL family genes in human kidney cancer and normal tissues were searched in GeneBank and 15 CXCL genes (CXCL1/2/3/4/5/6/8/9/10/11/12/13/14/16/17) were obtained. Oncomine and GEPIA analyses were performed to assess the expression levels of the 15 CXCL genes in KIRC vs. normal tissues. Eight CXCL genes (CXCL2/5/9/10/11/12/13/16) were differentially expressed ($p < 0.01$, Fig. 1A). In cancer tissues, seven genes (CXCL2/5/9/10/11/13/16) were up-regulated while CXCL12 was down-regulated. GEPIA was used to determine the correlation between gene expression levels and clinical stages. Four CXCL genes (CXCL5/9/10/11, Fig. 1B) exhibited significant correlations with clinical stages ($p < 0.01$). Oncomine was used to assess the expression levels of 8 CXCL genes in Pan-cancer (Fig. 1C).

The PPI network and functional enrichments

The top 100 genes that were correlated with the 8 target CXCL genes were obtained and an intersection of all correlation genes established. Fifty-five genes were obtained. The STRING database was used to construct the PPI network and to perform GO/KEGG enrichment analysis (Fig. 2A-B). From the PPI network, 13 genes were found to be closely associated with CXCL genes, such as, CCL5, CCL25, CCL27, and PF4. Enriched GO terms for the 8 CXCL genes were associated with: i. The chemokine-mediated signaling pathway (GO:0070098, GO:1990868, and GO:1990869) in biological processes; ii. The external side of the plasma membrane (GO:0009897), and secretory granule membrane (GO:0030667) in cellular components, and iii. Chemokine activities (GO:0008009) as well as chemokine receptor binding (GO:0042379) in molecular functions. KEGG analysis revealed that the CXCL genes were involved in viral protein interactions with cytokine and cytokine receptor (hsa04061) and Chemokine signaling pathway (hsa04062, Fig. 2C).

TIMER correlation analysis revealed that several of the eight CXCL genes, including CXCL9 and CXCL10, CXCL9 and CXCL11, CXCL10 and CXCL11 were strongly correlated (Fig. 2D-E).

Gene mutation analysis

CXCL2 and CXCL5 mutations and copy numbers were analyzed using the cBioPortal online tool. It was found that CXCL2 and CXCL5 were rarely mutated among KIRC patients (Fig. 3A). The likelihood of two CXCL genes having the same copy number was low. Then, we searched for SNP mutations, somatic mutations, DNA methylation, and copy number variations for the two CXCL genes in UCSC Xena(Fig. 3B). The findings were comparable to those obtained from the cBioPortal online tool.

Survival analysis

KM analysis revealed that only CXCL2 and CXCL5 had a survival value for KIRC patients ($p < 0.01$, Fig. 4A). ROC analysis showed that the one-year accuracy of CXCL2 and CXCL5 genes were 0.706 and 0.738, respectively (Fig. 4B-C). To assess the association between clinical information and target genes, univariate and multivariate Cox proportional hazard regression analyses were performed (Table 1).

In the univariate Cox analysis, all seven indices were positively correlated (T stage, N stage, M stage, pathology stage, age and CXCL2 as well as CXCL5 levels; $p < 0.01$). However, in multivariate Cox analysis, two indices (M stage and age) were positively correlated with target genes ($p < 0.05$).

In vitro analyses

In caki-1 cells, CXCL2 genes were upregulated while CXCL5 did not exhibit significant variations when compared to 293T cells (Fig. 5A). Moreover, WB analysis revealed differential CXCL2 protein levels between benign and malignant renal cells (Fig. 5B). Analysis of the three CXCL2 siRNAs showed that

siRNA-227 resulted in the lowest expressions of the CXCL2 gene. Therefore, caki-1 cells and caki-1 CXCL2 low cells (caki-1 cells mixed with siRNA-227) were used for subsequent experiments (Fig. 5C).

The CCK assay showed that caki-1 CXCL2 low cells had weaker proliferative capacities, compared to caki-1 cells while the Transwell assay confirmed that caki-1 CXCL2 low cells had worse migration and invasive capacities, compared to caki-1 cells (Fig. 5D-F). These findings imply that CXCL2 levels are upregulated in cancer cells and that CXCL2 expression levels affect cancer cell proliferation, migration as well as invasive capacities.

Upstream miRNA prediction and analysis

To identify the potential miRNAs upstream of CXCL2, 4 miRNA prediction databases, including miRDB, miRWalk, RNA22, and TargetScan were searched. Moreover, intersections of probable miRNAs were created using findings from the 4 databases. Six miRNAs upstream of CXCL2 were found.

Only one miRNA (hsa-miR-532-5p) was negatively correlated with CXCL2 ($p = 4.87E-06$, Fig. 6A), and was found to be down-regulated in KIRC tissues ($p < 0.001$, Fig. 6B). Elevated hsa-miR-532-5p levels were associated with longer survival outcomes among KIRC patients ($p = 0.003$, Fig. 6C).

Tumor-infiltrating immune cells

We investigated the association between CXCL2 levels and six immune cell components (B cells, CD4 T cells, CD8 T cells, Macrophages, Neutrophils, and DC cells) in KIRC patients using the TIMER database (Fig. 7A-C). CXCL2 levels were positively correlated with CD4 T cells and Neutrophils ($p < 0.001$), but were negatively correlated with B cells ($p < 0.01$). However, COX analysis or KM analysis did not establish the survival value for CXCL2 levels combined with the three immune cell components in KIRC patients.

The risk score model

Cox proportional hazard regression was used to construct a CXCL gene signature with survival values. Six CXCL genes (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL13) were obtained after univariate Cox regression analysis ($p < 0.01$, Table 2), while 3 CXCL genes (CXCL1, CXCL5, and CXCL13) were obtained after multivariate Cox regression analysis ($p < 0.05$, Table 3). The 3 CXCL genes (CXCL1, CXCL5, and CXCL13) were included in the survival signature and considered as the risk score (Fig. 7B). The risk score = (0.115681*Exp CXCL1) + (0.060388*Exp CXCL5) + (0.118217*Exp CXCL13). KM analysis revealed that KIRC patients with lower risk scores had significantly longer survival times. At the same time, the association between the risk score and other clinical information was assessed. There was a strong relationship between the risk score and T stage, pathological stage, and pathological grade.

Discussion

The tumor microenvironment is critical for tumor cell survival, progression, and metastasis. CXCL genes are involved in autophagy-mediated injury and cell inflammation. Moreover, CXCL gene levels have an impact on the tumor microenvironment(25). However, the association between CXCL genes and KIRC outcomes has not been conclusively determined.

In this study, among the 14 CXCL genes, only eight CXCL genes were differentially expressed between cancer and normal tissues. These CXCL genes were established to play a role in the development and progression of several cancer types, including KIRC.

Functional enrichment analysis revealed that these CXCL genes and their correlation genes were mainly enriched in chemokine activities and chemokine receptor binding in KIRC. Similar findings have been reported in other cancer studies(26, 27).

From the 8 differentially expressed CXCL genes, only CXCL2 and CXCL5 were found to have a significant survival value in KIRC patients. Therefore, there is a need to investigate the significance and mechanisms of CXCL2 and CXCL5 in KIRC patients. In vitro, CXCL2 was proven to be upregulated in renal cancer cell. The cell functional experiment confirmed that CXCL2 levels exerted various effects on renal cancer cell proliferation, migration and invasion. These findings strongly confirm that CXCL2 may have a survival value for KIRC patients. There were no significant differences in CXCL5 expression levels between 293T cells and caki-1 cells. We postulated that stromal cells of renal carcinoma secrete the CXCL5 protein, which may have adverse effects on survival outcomes for KIRC patients.

Studies are assessing the association between tumor-secreted chemokines and the tumor immune environment. In this study, CXCL2 levels were strongly correlated with CD4 T cells, B cells, and neutrophils. However, there was no direct evidence that the three immune cell components have survival values for KIRC patients. But they have important immune function regulatory effects. CXCL2, as a chemokine, can be secreted by tumor cells or other immune cells. The CXCL2 protein secreted from M2 polarized macrophages promoted hepatocellular carcinoma cell proliferation and migration(28). In this study, elevated CXCL2 levels in renal cells were associated with better proliferation and migration abilities. With regards to the effects of CXCL2 proteins on renal carcinoma cell functions, it was not established whether immune cells are involved in the respective mechanisms. In the future, we would further study the mechanism between CXCL2 protein and immune cells in the KIRC patients.

Conclusion

In KIRC patients, 8 CXCL genes were differentially expressed, however, only CXCL2 and CXCL5 had a significant survival value. Upregulated CXCL2 promotes renal cancer cell progression.

Abbreviations

KIRC
kidney Clear Cell Carcinoma

PPI
the protein-protein interaction
KM
Kaplan Meier
Q- PCR
R- Quantitative real-time polymerase chain reaction
WB
western blot

Declarations

Ethics approval and consent to participate

We confirmed that all the methods in our paper were performed in accordance with the relevant guidelines of the helsinki and our research was approved by the ethics committee of our hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and institutional requirements.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analysed during the current study are available in the TCGA-KIRC repository, [https://xenabrowser.net/datapages/].

Competing interests

we declared that the authors have no competing interests as defined by BMC, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Authors' contributions

JS wrote the draft manuscript; RW and JT edited the manuscript; YC, ZF, JY, XC, and JG analyzed the data; SX developed the images. JS also did the experiment. All authors revised the manuscript and approved the submitted version.

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Tables

Table 1: the cox survival analysis for the CXCL2 and other clinical indexes in the KIRC dataset

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
T stage	539				
T1	278	Reference			
T2&T4&T3	261	2.917 (2.095-4.061)	**<0.001**	0.563 (0.159-1.999)	0.374
N stage	257				
N0	241	Reference			
N1	16	3.453 (1.832-6.508)	**<0.001**	1.717 (0.832-3.544)	0.144
M stage	506				
M0	428	Reference			
M1	78	4.389 (3.212-5.999)	**<0.001**	3.062 (1.885-4.973)	**<0.001**
Pathologic stage	536				
Stage I	272	Reference			
Stage II&Stage III&Stage IV	264	3.299 (2.342-4.648)	**<0.001**	3.176 (0.840-12.002)	0.088
Age	539				
<=60	269	Reference			
>60	270	1.765 (1.298-2.398)	**<0.001**	1.873 (1.223-2.868)	**0.004**
CXCL2	539				
Low	269	Reference			
High	270	1.539 (1.136-2.085)	**0.005**	1.285 (0.817-2.020)	0.278
CXCL5	539				
Low	269	Reference			
High	270	1.596 (1.177-2.163)	**0.003**	1.352 (0.855-2.137)	0.197

Table 2: the univariate Cox proportional hazard regression of CXCL genes on the KIRC patients

Gene ID	HR	HR.95L	HR.95H	P value
CXCL1	1.181704	1.110081	1.257949	1.66E-07
CXCL5	1.12314	1.07325	1.175351	5.46E-07
CXCL2	1.224121	1.128515	1.327826	1.09E-06
CXCL13	1.149988	1.08359	1.220455	4.11E-06
CXCL3	1.230473	1.123092	1.348122	8.52E-06
CXCL6	1.101454	1.047003	1.158736	0.000187

Table 3: the multivariate Cox proportional hazard regression of CXCL genes on the KIRC patients

Gene id	coef	HR	HR.95L	HR.95H	P value
CXCL1	0.115681	1.122638	1.043586	1.207678	0.001902
CXCL13	0.118217	1.125488	1.060333	1.194647	0.000102
CXCL5	0.060388	1.062249	1.007414	1.120069	0.025542

Figures

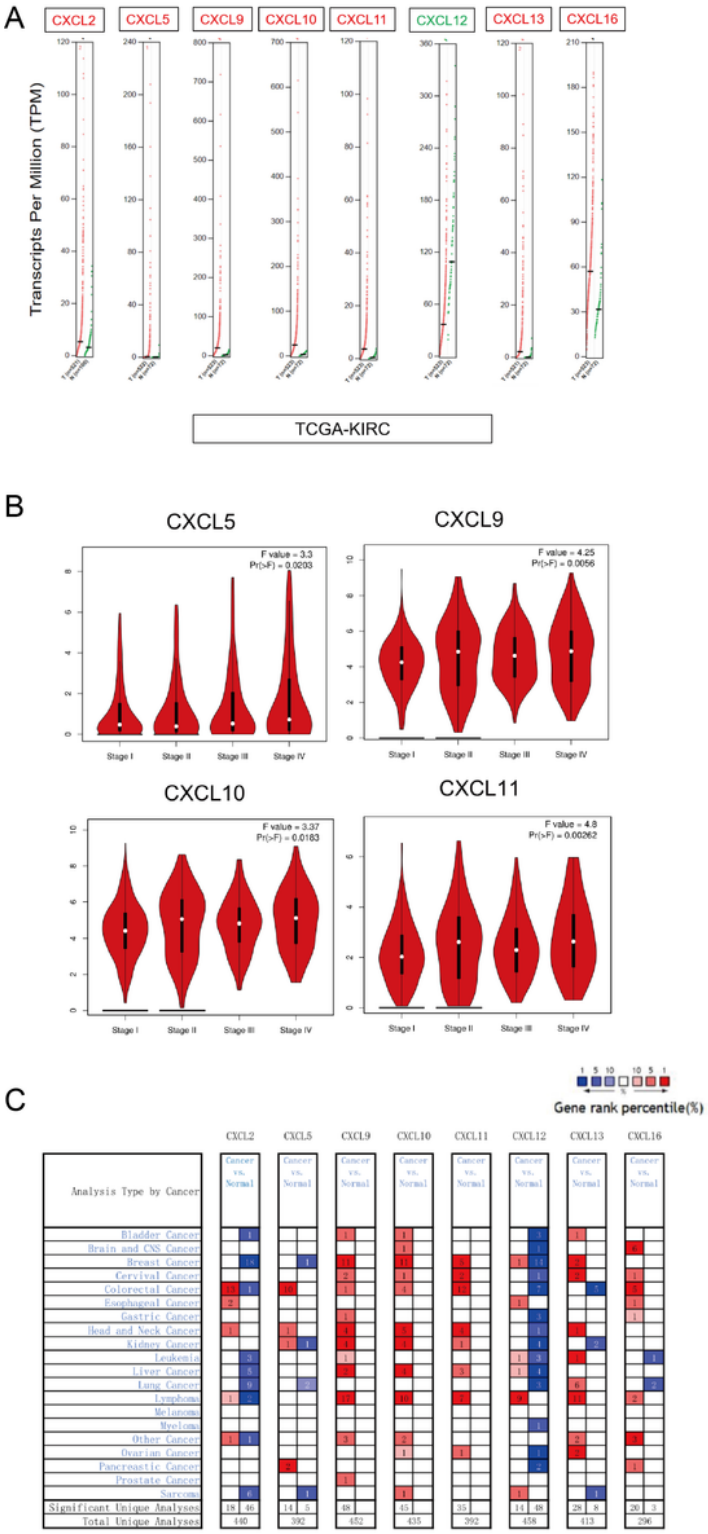
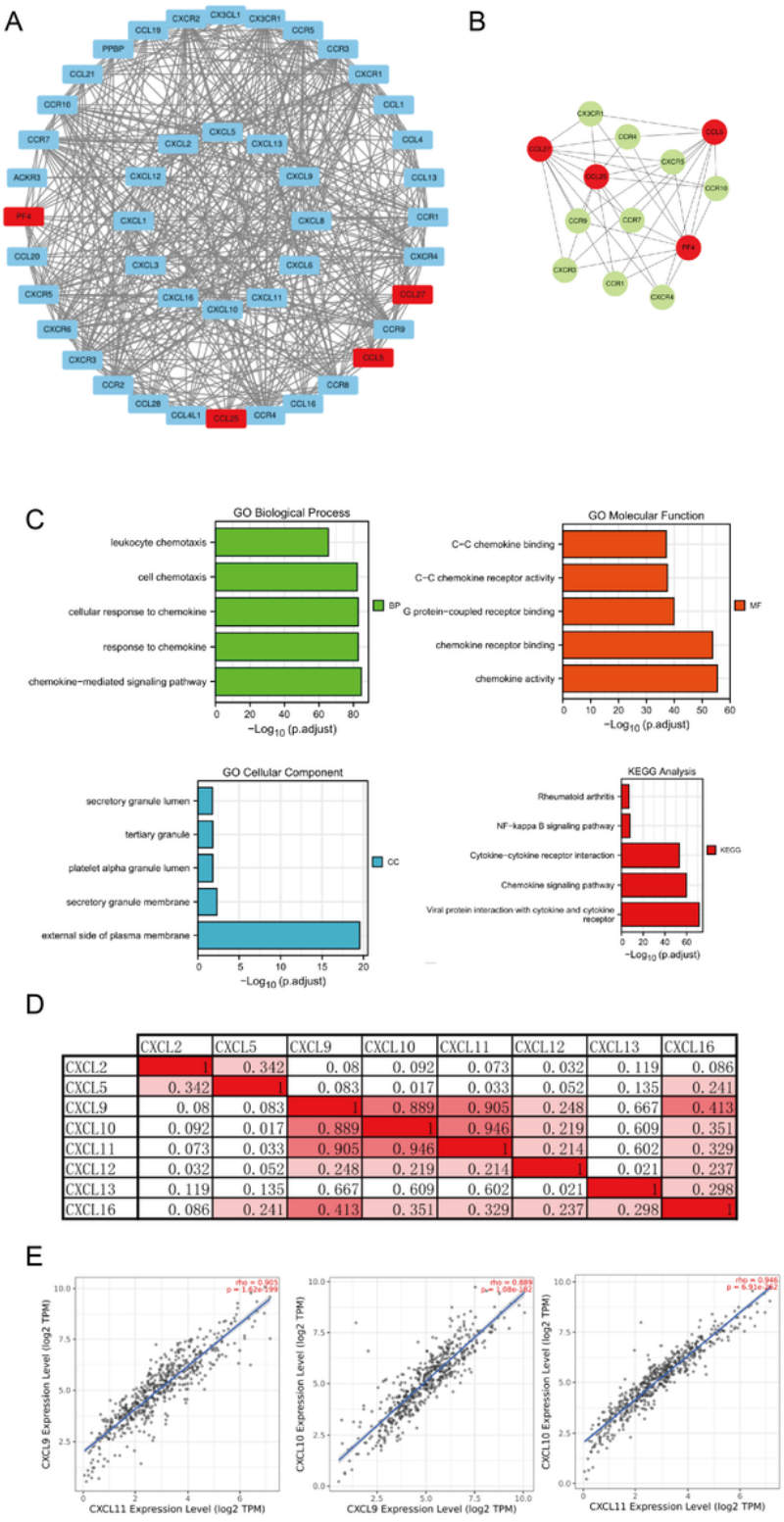


Figure 1

A the expression level of 8 CXCL genes in the KRC dataset; B clinical stage of 4 CXCL genes in the KIRC dataset; C the expression level of 8 CXCL genes in the Oncomine.



	CXCL2	CXCL5	CXCL9	CXCL10	CXCL11	CXCL12	CXCL13	CXCL16
CXCL2	1	0.342	0.08	0.092	0.073	0.032	0.119	0.086
CXCL5	0.342	1	0.083	0.017	0.033	0.052	0.135	0.241
CXCL9	0.08	0.083	1	0.889	0.905	0.248	0.667	0.413
CXCL10	0.092	0.017	0.889	1	0.946	0.219	0.609	0.351
CXCL11	0.073	0.033	0.905	0.946	1	0.214	0.602	0.329
CXCL12	0.032	0.052	0.248	0.219	0.214	1	0.021	0.237
CXCL13	0.119	0.135	0.667	0.609	0.602	0.021	1	0.298
CXCL16	0.086	0.241	0.413	0.351	0.329	0.237	0.298	1

Figure 2

A the PPI network of CXCL genes; B the hub genes of CXCL genes PPI network; C GO and KEGG analysis of CXCL genes; D the correlation of CXCL genes; E the positive correlation of CXCL genes.

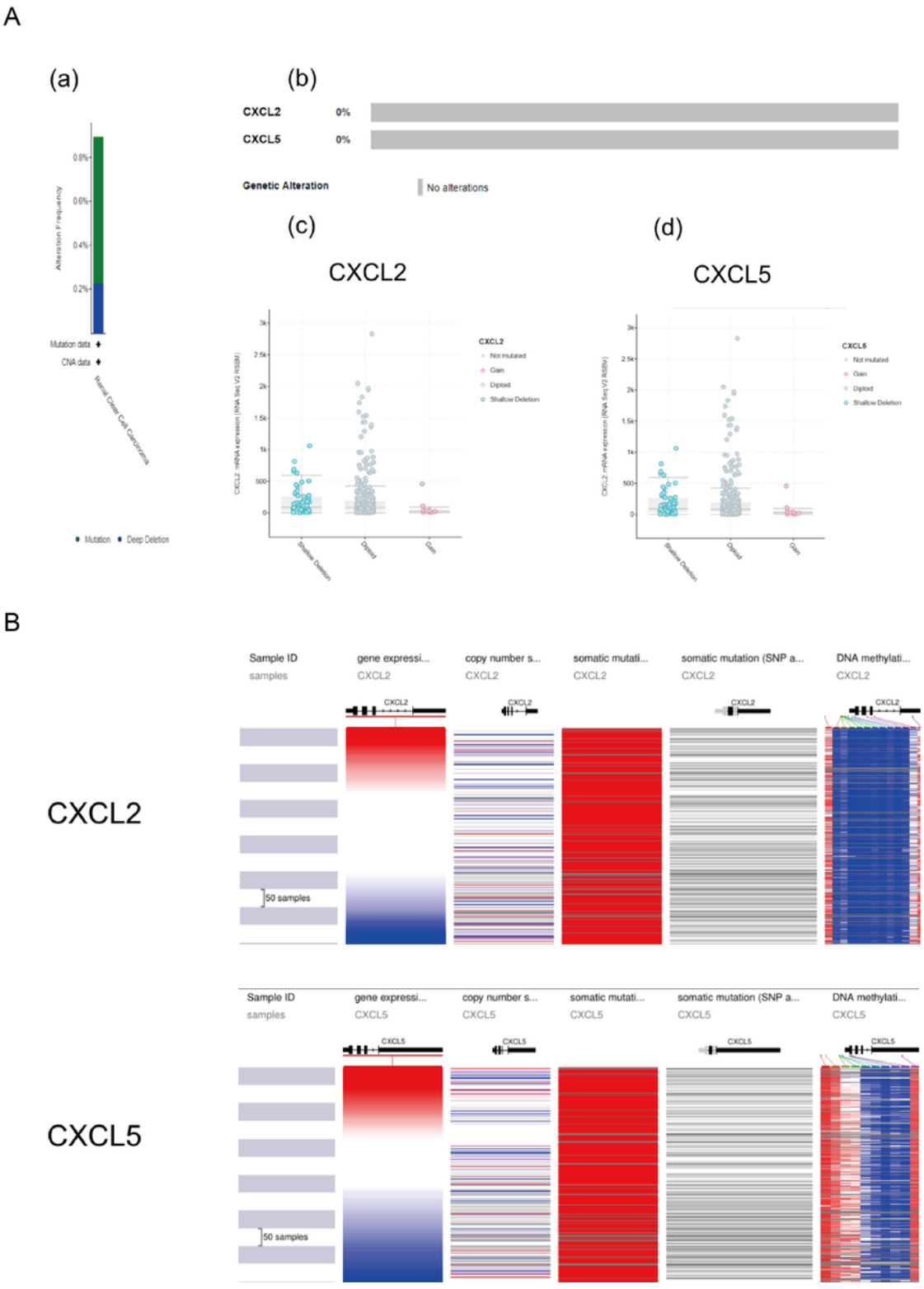


Figure 3

A (a-b) the mutation of CXCL2 and CXCL5 in the website of cBioPortal; A (c-d) the copy number of CXCL2 and CXCL5 in the website of cBioPortal; B the expression, copy number, somatic mutation, SNP, and DNA methyation of CXCL 2 and CXCL5 in the website of UCSC Xena.

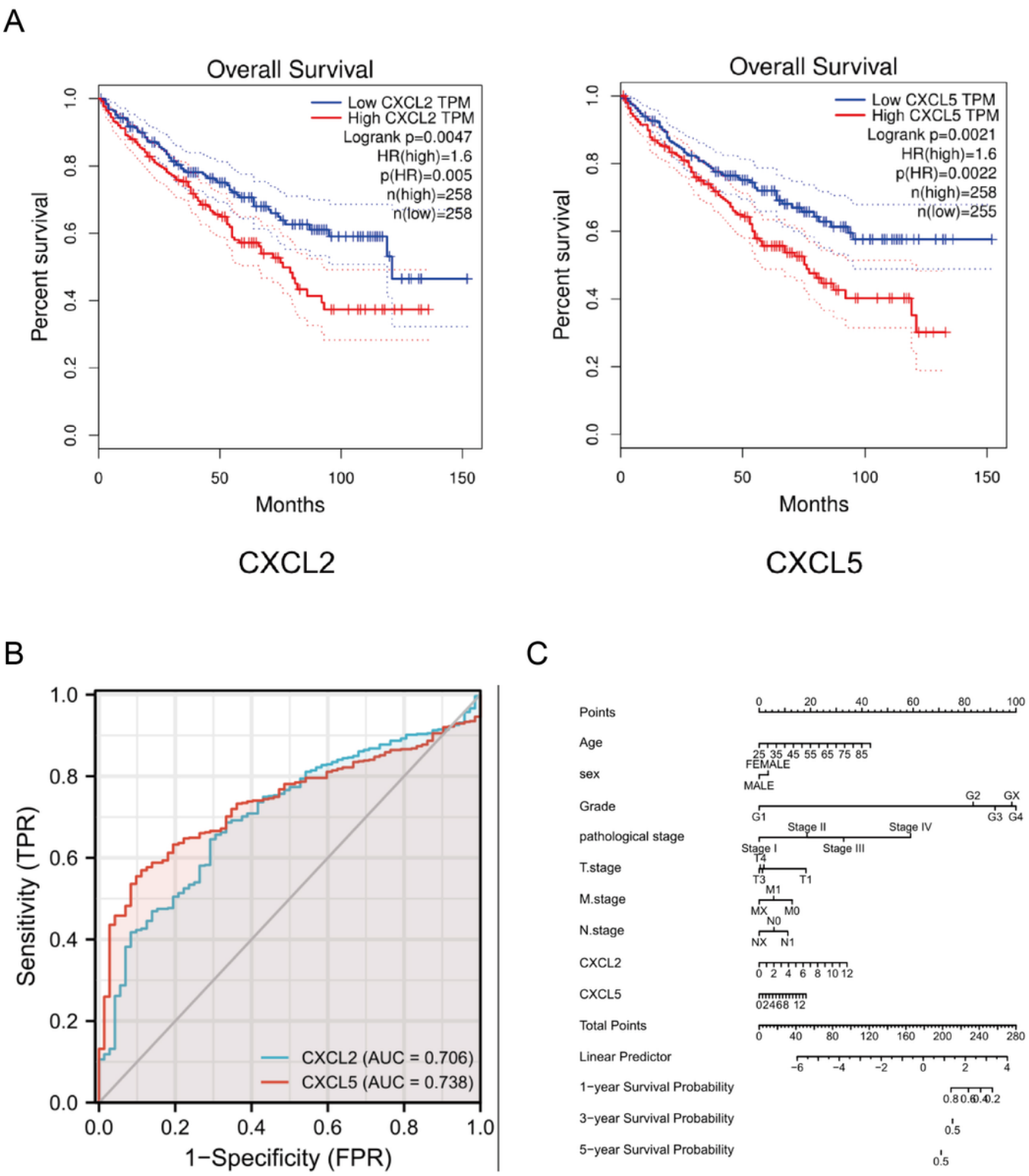


Figure 4

A the KM analysis of CXCL2 and CXCL5; B ROC time dependent analysis of CXCL gens; C the nomogram of CXCL genes and clinical information.

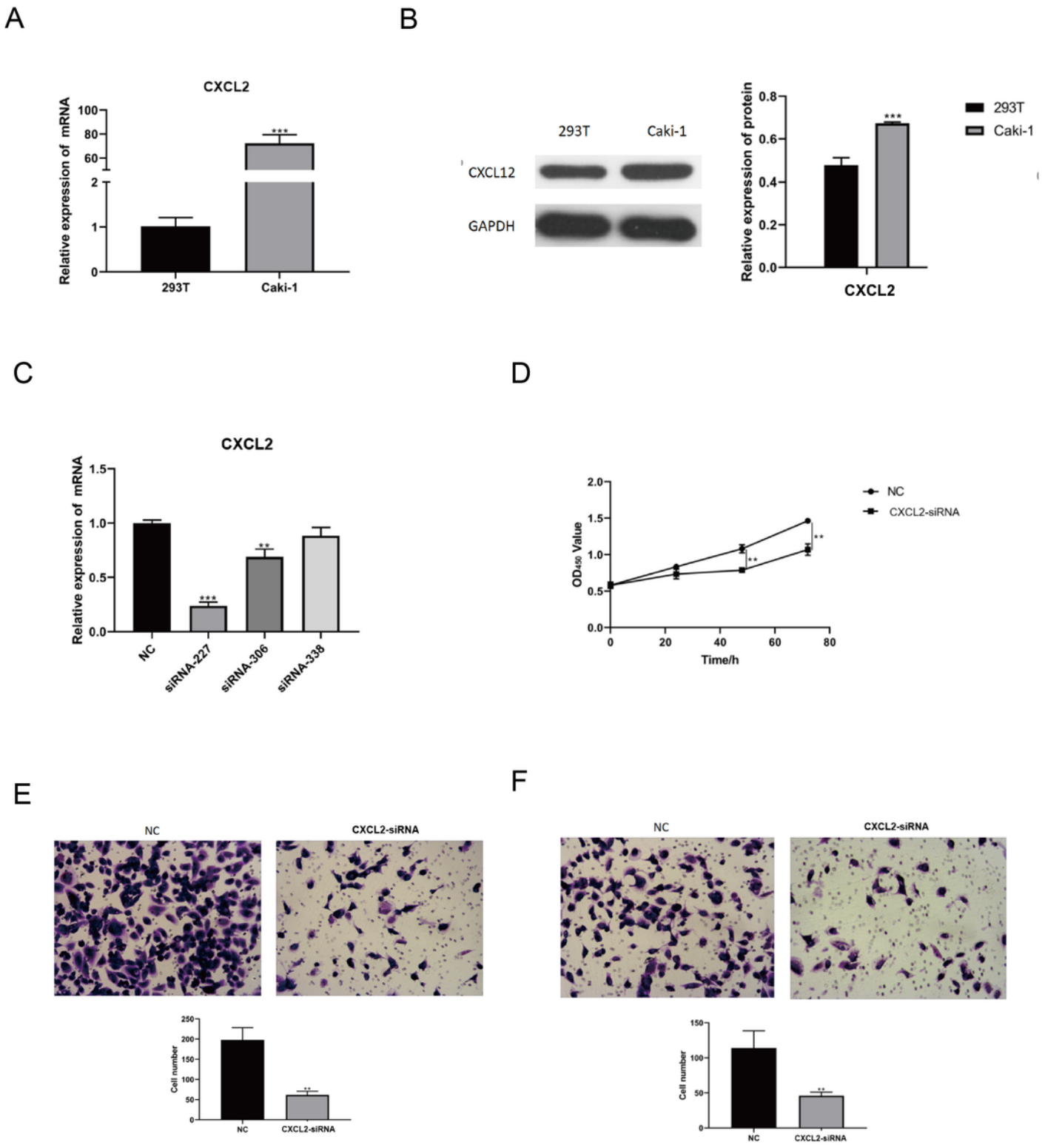


Figure 5

A-B the expression of CXCL2 between caki-1 cell line and 293T cell line in the Q-PCR or WB; C the expression of CXCL2- siRNA in the Q-PCR; D: the results comparison between CXCL2- siRNA and NC cell line in the CCK8 assay; E-F the results comparison between CXCL2- siRNA and NC cell line in the migratory and invasive capacity.

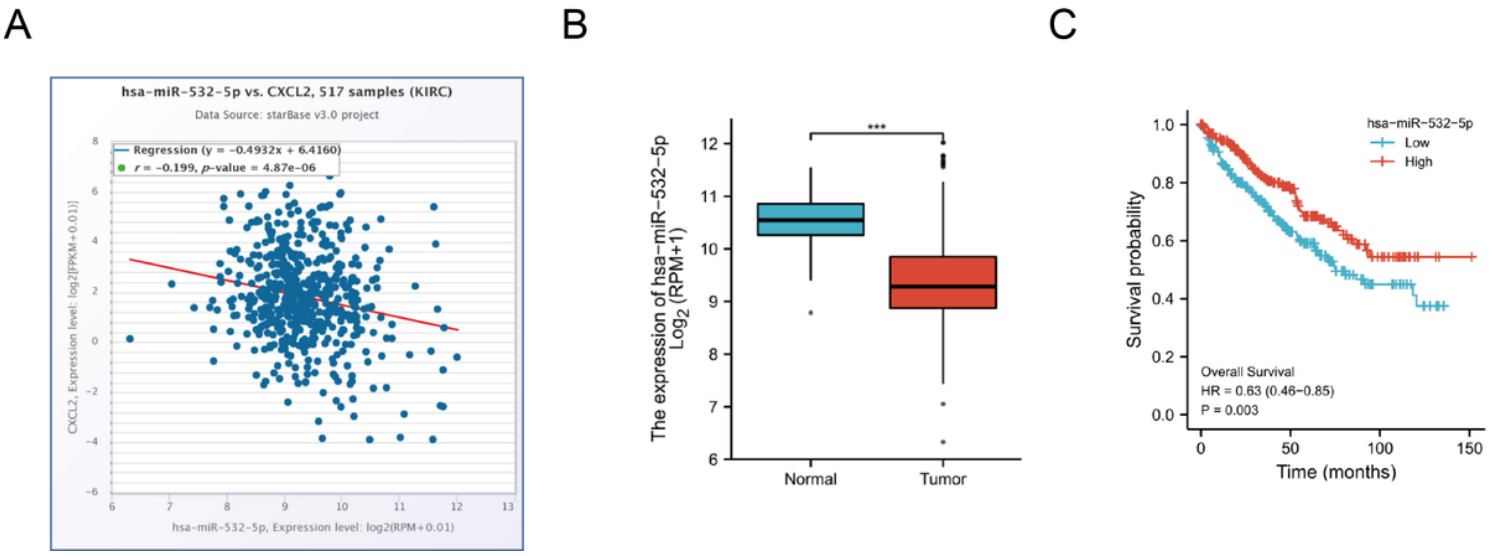


Figure 6

A the correlation of CXCL2 and hsa-miR-532-5p; B the expression of hsa-miR-532-5p in the KIRC patients; C the KM analysis of hsa-miR-532-5p in the KIRC patients.

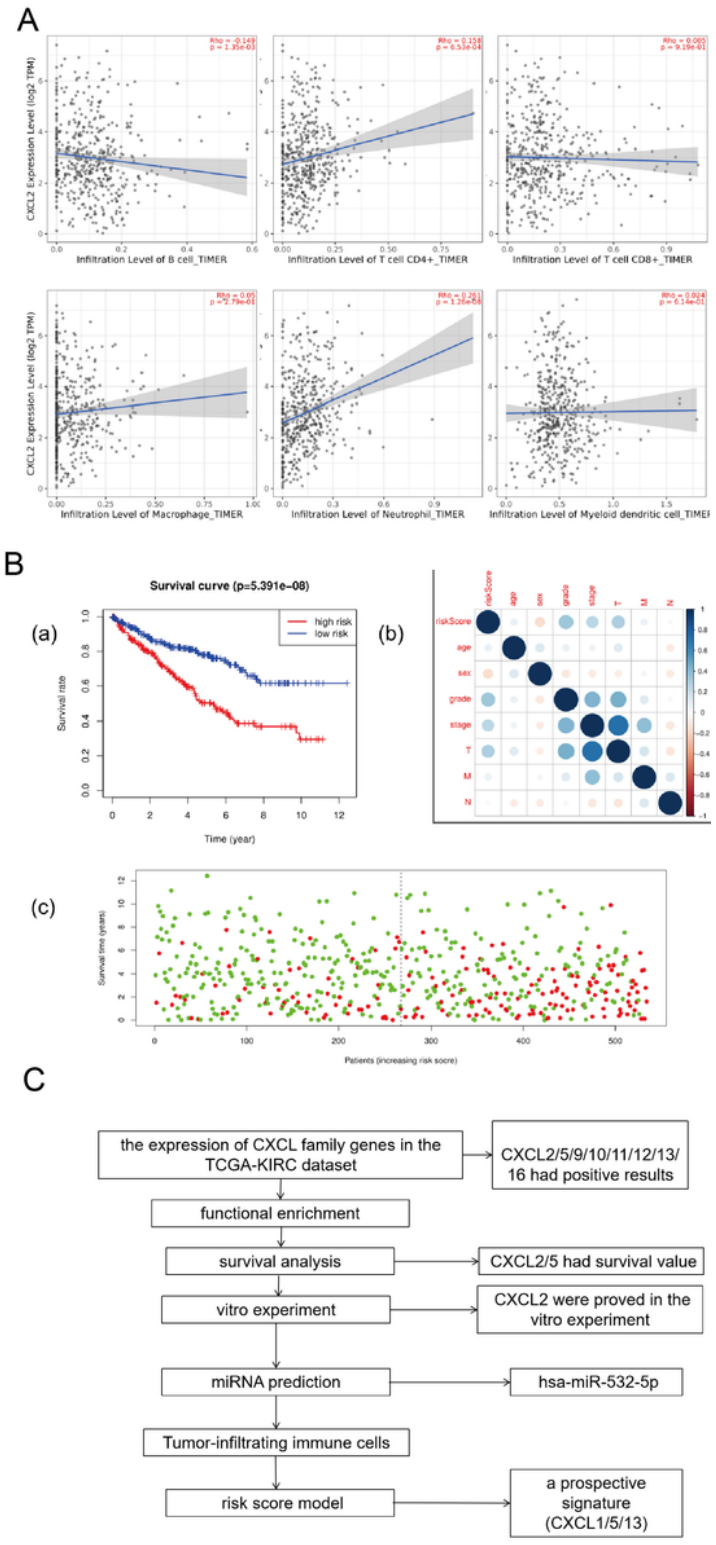


Figure 7

A the correlation of CXCL2 and 6 immune cell component from the TIMER; B: the KM analysis and correlation of risk score; C: the flow chart.