Identification of Autophagy-Related Long Non-Coding RNA Prognostic Signature for Clear Cell Renal Cell Carcinoma

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Research

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Abstract

Background: Studies over the past decade have shown that long non-coding RNAs (lncRNAs) play an essential role in the tumorigenesis and progression of kidney renal clear cell carcinoma (KIRC). Meanwhile, autophagy has been demonstrated to regulate KIRC pathogenesis and targeting therapy resistance. However, the prognostic value of autophagy-related lncRNAs in KIRC patients has not been reported before.

Methods: In this study, we obtained transcriptome data of 611 KIRC cases from the TCGA database and 258 autophagy-related mRNAs from the HADb database to identify autophagy-related lncRNAs by co-expression network. A prognostic model was then established basing on these autophagy-related lncRNAs, dividing patients into high-risk and low-risk groups. Survival analysis, clinical variables dependent receiver operating characteristic (ROC) analyses, univariate/multivariate Cox analyses, and clinical correlation analysis were performed based on risk signature with R language. Gene set enrichment analysis (GSEA) was then performed to investigate the potential mechanism of the risk signature promoting KIRC progression with GSEA software.

Results: A total of 17 lncRNAs were screened out and all these lncRNAs were found significantly related to KIRC patients' overall survival in subsequent survival analyses. Besides, the overall survival time in the high-risk group was much poorer than in the low-risk group. The ROC analysis revealed that the prognostic value of risk signature was better than age, gender, grade, and N stage. Univariate/multivariate analyses suggested that the risk signature was an independent predictive factor for KIRC patients. Immune-related pathways were dramatically enriched in high-risk patients according to GSEA.

Conclusions: In summary, our identified 17 autophagy-related lncRNAs had prognostic value for KIRC patients which may function in Immunomodulation.

Introduction

Renal cell carcinoma (RCC) occupied approximately 3% of all cancers, with an annual increase of 2% incidence, leading to more than 400,000 new cases and 175,098 deaths worldwide in 2018 [1]. Kidney renal clear cell carcinoma (KIRC) is a rather aggressive subtype representing about 85% of metastatic RCC cases and 67% of all stage RCC [2]. Benefiting from the development of modern surgery and targeted drug research, most early-stage KIRC patients have a relatively high 5-year survival rate. However, approximately 25–30% of cases accompany metastases at diagnosis and 20–30% of patients show relapse after undergoing surgical management for local RCC [3], making the 5-year survival rate drop to only 23% [4]. Lacking of reliable and stable prognostic markers bears responsibility for that. Presently, the clinical prognosis of KIRC patients is predicted by multidimensional factors, including clinical, anatomical, molecular factors, and histological with non-reliable guideline-approved biomarkers [5]. For molecular factors, CAIX, PTEN, and CXCR4, etc. have been investigated one after another, however none of which has yet improved the current prognostic systems [6].

Protein-coding RNAs account for only 1%-2% of the human genome and more than 90% of RNAs are thought to carry non-protein-coding information with other functions, modifying or regulating, etc [7]. During the past decade, the role of lncRNAs in RCC has been clearly highlighted. HOTAIR which is elevated in RCC cells is one of the few well-described lncRNAs that could be further investigated as a reliable molecular marker and promising target for RCC patients [8]. Likewise, H19 is considered as an oncogene in RCC which plays an essential role in the epithelial-to-mesenchymal transition (EMT) process by mediating the function of EZH2, E-cadherin, and β-catenin [9]. In addition to tumorigenesis and progression, lncRNA was also demonstrated to regulate drug resistance in RCC. SRLR [10], ARSR [11], and NEAT1 [12] were believed to promote drug resistance. In contrast, GAS5 was known to enhance RCC cell's sensitivity to sorafenib via the GAS5/miR-21/SOX5 axis [13].

Since first reported 40 years ago, autophagy is involved in the pathologic process of various diseases, including cancer [14], cardiovascular disease [15], infection, and immune deficiency [16]. Recently, the association between autophagy and RCC has been studied in signaling pathways and drug sensibility. For example, autophagy in RCC cells can be inhibited by the

activation of the PI3K/AKT/mTOR axis, accompanied by protein translation and cell proliferation [17]. Besides, sunitinib was demonstrated to cause autophagy in RCC cells by inhibiting AKT/mTOR signaling pathway [18].

In this work, we identified 17 autophagy-related lncRNAs through bioinformatics methods. Following prognosis analyses revealed that these lncRNAs were significantly associated with KIRC patients' survival. Finally, immune-related pathways were dramatically enriched in high-risk groups according to GSEA results.

Methods

Workflow

The steps we used in this work to screen autophagy-related lncRNAs and establish a prognosis model for KIRC were showed in Fig. 1.

Data Collection And Pre-processing

We downloaded transcriptome profiling of KIRC from TCGA (https://portal.gdc.cancer.gov/) and selected HTseq-FPKM. A total of 611 cases were included in our subsequent analyses. We next separated RNA to mRNA and lncRNA using human.gtf downloaded from Ensembl database (http://asia.ensembl.org/index.html). Autophagy related mRNA gene list was downloaded from Human Autophagy Database (HADb, http://www.autophagy.lu/index.html). By merging this gene list and mRNA expression matrix, we got the autophagy-related mRNA gene expression. Clinical information was also obtained from TCGA and 537 cases were included for prognostic analysis.

Co-expression Network

To screen autophagy-related lncRNA, we constructed mRNA-lncRNA co-expression network by using the R language. The correlation coefficient $|R|>0.3$ and p value < 0.0001 were considered significantly correlated. The co-expression network between autophagy-related mRNA and lncRNA was mapped in Cytoscape (v3.7.2).

Risk Score Calculation

First, clinical data and autophagy-related lncRNA expression were combined to get the relationship between expression and survival time. We then performed univariate and multivariate Cox regression analyses to evaluate the prognostic value of these lncRNAs. The lncRNAs with a p-value < 1E-6 by univariate analysis were further enrolled to conduct multivariate Cox regression analysis to assess the risk score. The Akaike information criterion (AIC) values were used to optimize the Cox model and the lncRNAs with the lowest AIC were retained in the final signature. The risk score formula was as followed: Risk score= $\sum \mathrm{lncRNAi}_{exp} \times \mathrm{lncRNAi}_{coef}$ ($\mathrm{lncRNAi}_{exp}$ indicates the expression of every single lncRNA and the $\mathrm{lncRNAi}_{coef}$ was calculated using a multivariable Cox proportional hazards model). All patients were divided into high or low-risk group basing on the median risk score. Sankey diagram was built with the "ggalluvial" package according to the HR (hazard ratio) value of multivariate Cox proportional hazards regression analyses (HR > 1 was considered as risk gene, HR < 1 was considered as protect gene.).

Prognosis Model Construction

Given the prognosis related lncRNAs, we subsequently constructed a prognosis model to assess the prognostic value of these lncRNAs for KIRC patients. The overall survival (OS) curves were plotted with "survival" package basing on the

expression of lncRNAs or risk score. The independence of risk signature for prognosis was further evaluated by univariate and multivariate Cox proportional hazards regression analyses. For a more intuitive understanding of the relevance between lncRNAs expression and patients' survival state, we drew the risk score curve, survival time, and heatmap. The heatmap was drawn with "pheatmap" packages. To evaluate the signature's sensitivity and specificity, the clinical variables dependent receiver operating characteristic (ROC) analyses were performed with "survivalROC" package. Additionally, to explore the correlation between clinical features and risk score, we excluded patients with deficient information and 246 patients were retained for analysis.

Gene set enrichment analysis (GSEA) and immune cell infiltration analysis

611 KIRC samples in TCGA were divided into two groups (high risk and low risk) based on the median of risk scores in lncRNA signature. We conducted GSEA between the two groups to identify the significantly altered Gene Ontology (GO) pathways by using GSEA software (v4.0.3). C5.all.v6.1.symbols.gmt was used as the gene set. The P and FDR q values were obtained from 1,000 permutations and $P < 0.05$ was considered statistically significant. The correlation between the expression of lncRNAs and the immune cell infiltration level in KIRC was analyzed with the lmmLnc database (http://bio-bigdata.hrbmu.edu.cn/lmmLnc/index.jsp). B cell, T cell regulatory (Tregs), T cell NK, T cell CD4 + Th1, T cell CD8 and Neutrophil were included.

Statistical analysis

Almost all analyses were performed with R software (4.0.0) or Perl (5.32.0). The Student's t-test was used to compare the differences between two or three groups. P value < 0.05 was considered statistically significant.

Results

17 lncRNAs were identified as autophagy-related

A total of 14142 lncRNAs were distinguished from the transcriptome matrix downloaded from the TCGA database. 258 autophagy-related mRNAs were obtained from the HADb database. The co-expression network was constructed to identify autophagy-related lncRNAs. Finally, 17 lncRNAs and 99 autophagy-related mRNA were screened out and considered as significant correlation (table S1, $|R|>0.3$, p value < 0.0001). We then used these RNAs as nodes to draw a network (Fig. 2a), the red ellipse indicating lncRNA, blue ellipse indicating autophagy-related mRNA, and the line between them indicating the co-expression relationship. Furthermore, we created this image (Fig. 2b)—a Sankey diagram—by plotting the connection within each node and connecting them to risk type which based on the HR value of multivariate Cox proportional hazards regression analyses (HR > 1 was considered as risk gene, HR < 1 was considered as protecting gene. table s2). Three of them were defined as protect genes (AC121338.2, EPB41L4A-DT, LINC01843) and others were all risk genes (AL391244.3, AC011462.4, AC103706.1, SNHG15, AL590094.1, AP003352.1, AC026356.2, SNHG17, LINC00460, HOTAIRM1, AC084876.1, AC027796.4, MELTF-AS1, AC010973.2).

Survival Analyses Of These 17 Autophagy-related Lncrnas

Given the 17 autophagy-related lncRNAs, we next explored their impact of expression level on OS (Fig. 3). For three protective autophagy-related lncRNAs, they showed a significant tendency that higher expression predicting better survival rate. In contrast, for 14 risky autophagy-related lncRNAs, higher expression may lead to poorer survival rates. The survival analysis results were consistent with the risk type classified by multivariate Cox proportional hazards regression analyses.

Risk Signature Assessment

After performing survival analyses of these autophagy-related lncRNAs, we next constructed the prognosis assessment of risk signature. First, we divided patients into high-risk group and low-risk group to compare their survival difference. As shown in Fig. 4a, the high-risk group displayed a lower survival rate than the low-risk group. Next, we plotted the expression patterns of the 17 autophagy-related lncRNAs, survival status dot plot, and risk score curve to visualize the association between these variables (Fig. 4b). The heatmap showed that 3 protective autophagy-related lncRNAs (LINC01843, EPB41L4A-DT, and AC121338.2) were down-regulated in high-risk group whereas other lncRNAs were overexpressed. Additionally, the survival status dot plot revealed that dead cases were almost concentrated in the high-risk group and more dead cases appeared with the increase of risk score.

In the ROC analysis (Fig. 4c), the prognostic model was evaluated using the area under the curve (AUC). The AUC for the risk score signature was 0.748, higher than age (0.580), gender (0.480), grade (0.743) and N stage (0.527, lymph node metastasis), but lower than stage (0.846), T stage (0.788, the extent of the primary tumor) as well as M stage (0.751, distant metastasis). The results suggested that the prognostic value of risk signature was better than age, gender, grade, and N stage.

For the independent prognosis analysis, age, gender, grade, stage, TNM system, and risk score were included in the independent prognostic factors. Univariate (Fig. 4d) and multivariate (Fig. 4e) analyses suggested that the risk signature was an independent predictive factor for KIRC patients ($p < 0.001$, HRs were 1.112 and 1.088, respectively). The details of analysis were shown in Table 1 and Table 2.

Table 1
Univariate Cox analysis of characteristics and risk score in KIRC.

id	B	SE	z	HR	HR.95L	HR.95H	pvalue
age	0.023401	0.009062	2.582371	1.023677	1.005656	1.042021	0.009812**
gender	0.017341	0.213742	0.081132	1.017492	0.669258	1.546923	0.935337
grade	0.757602	0.143102	5.294148	2.133156	1.61144	2.823781	1.20E-07***
stage	0.59752	0.097529	6.126595	1.817605	1.501353	2.200474	8.98E-10***
T	0.614276	0.119009	5.161601	1.848318	1.463781	2.333873	2.45E-07***
M	1.391904	0.222367	6.259497	4.022501	2.601461	6.219781	3.86E-10***
N	1.110114	0.336707	3.296975	3.034706	1.568596	5.871135	0.000977***
riskScore	0.106298	0.015117	7.031483	1.112153	1.079684	1.145598	2.04E-12***

**$p < 0.01$; ***$p < 0.001$.

Table 2
Multivariate Cox analysis of characteristics and risk score in KIRC.

id	B	SE	z	HR	HR.95L	HR.95H	pvalue
age	0.037606	0.010293	3.653502	1.038322	1.017585	1.059482	0.000259***
gender	0.262189	0.228932	1.145275	1.299773	0.829852	2.035796	0.252095
grade	0.269428	0.172746	1.559684	1.309216	0.93319	1.836761	0.118835
stage	0.416601	0.264258	1.57649	1.516797	0.903629	2.546036	0.114913
T	-0.14512	0.250034	-0.5804	0.86492	0.529843	1.411903	0.561648
M	0.509551	0.416789	1.222565	1.664544	0.735401	3.767613	0.221494
N	0.428635	0.362005	1.184059	1.535161	0.755118	3.120992	0.23639
riskScore	0.084718	0.018718	4.526048	1.088411	1.049204	1.129082	6.01E-06***

***p < 0.001.

Clinical Value Of The Risk Signature For Kirc Patients

Subsequently, we evaluated the clinical value of the risk signature regarding age, gender, grade, stage, and TNM. As showed in Table 3, the risk score tended to elevate in higher grade (grade 3–4, p = 0.0001), advanced stage (stage Ⅲ-Ⅳ, p = 0.0001), higher T stage (p = 0.001) and N stage (p = 0.04). These results suggested that the risk signature might be associated with the progression of KIRC.

Table 3
Correlations between risk score signature and clinical features in the TCGA cohort.

Risk score						
Clinical	Group	n	Mean	SD	t	P
age	<=65	155	1.977	4.47	0.614461	0.54
	> 65	91	1.719	2.088		
gender	Female	97	1.803	2.73	-0.28856	0.773
	Male	149	1.933	4.316		
grade	G1-2	109	0.973	0.87	-3.84431	0.0001***
	G3-4	137	2.605	4.873		
stage	Stage -	133	1.036	0.978	-3.64565	0.0001***
	stage -	113	2.878	5.294		
T	T1-2	145	1.098	1.045	-3.40555	0.001**
	T3-4	101	3.007	5.566		
M	M0	205	1.463	1.899	-2.0149	0.051
	M1	41	3.976	7.94		
N	N0	232	1.659	3.442	-2.27093	0.04*
	N1-3	14	5.581	6.406		
*p < 0.05; **p < 0.01; ***p < 0.001.						

Identified autophagy-related lncRNAs may function in tumor progression via Immunomodulation

To explore the potential mechanism of these 17 autophagy-related lncRNAs' effect on tumor progression, we conducted Gene Ontology (GO) functional annotation with GSEA. Interestingly, immune-related pathways were markedly enriched in the high-risk group, including immune response mediated by circulating immunoglobulin, interferon-gamma production, regulatory T-cell differentiation, positive regulation of interferon-gamma production, immunoglobulin complex, B cell mediated immunity, regulation of humoral Immune response, and immunoglobulin receptor binding (Fig. 5a). The detailed enrichment results were summarized in Table 4. Given the pathway enriched in immune regulation, we next conducted analyses of the correlation between the expression of these lncRNAs and immune cells infiltration level in KIRC (Fig. 5b). The results revealed that the infiltration of T cell regulatory (Tregs), T cell NK and T cell CD4 + Th1 had a significantly positive correlation with lncRNAs expression. In contrast, the infiltration of neutrophil exhibited a negative correlation with the expression of lncRNAs.

Table 4
Gene set enrichment analysis (GSEA) results based on the risk signature of 14 autophagy related lncRNAs.

NAME	SIZE	ES	NES	P	FDR q-val	FWER p-val	RANK AT MAX	LEADING EDGE
GO_HUMORAL_IMMUNE_RESPONSE_MEDIATED_BY_CIRCULATING_IMMUNOGLOBULIN	139	0.600492	2.296511	0	0	0	18013	tags = 77%, list = 33%, signal = 114%
GO_INTERFERON_GAMMA_PRODUCTION	108	0.596715	2.199109	0	0	0	8083	tags = 47%, list = 15%, signal = 55%
GO_REGULATORY_T_CELL_DIFFERENTIATION	31	0.761541	2.293867	0	0	0	5855	tags = 71%, list = 11%, signal = 79%
GO_POSITIVE_REGULATION_OF_INTERFERON_GAMMA_PRODUCTION	64	0.655313	2.235942	0	0	0	7431	tags = 55%, list = 13%, signal = 63%
GO_IMMUNOGLOBULIN_COMPLEX	65	0.602932	2.09714	0	3.13E-04	0.006	14405	tags = 65%, list = 26%, signal = 87%
GO_B_CELL_MEDIATED_IMMUNITY	208	0.511539	2.055525	0	5.55E-04	0.015	14596	tags = 59%, list = 26%, signal = 80%
GO_REGULATION_OF_HUMORAL_IMMUNE_RESPONSE	130	0.543822	2.056492	0	6.06E-04	0.015	18640	tags = 77%, list = 34%, signal = 116%
GO_IMMUNOGLOBULIN_RECEPTOR_BINDING	65	0.595271	2.021653	0	0.00122	0.04	17569	tags = 71%, list = 32%, signal = 104%

ES, enrichment score; NES, normalized enrichment score. FDR, false discovery rate; FWER, familywise-error rate;

Discussion

LncRNA/miRNA/mRNA axis is a promising target for tumor treatment. Given the important role of lncRNAs and autophagy in KIRC tumorigenesis, progression, and drug resistance, we performed this work to screen autophagy-related lncRNAs and

assess their prognostic value for KIRC patients.

Among our identification of lncRNAs, several have been demonstrated to function in KIRC or other malignant tumors. Small nucleolar RNA host gene 15 (SNHG15) was upregulated in KIRC, knockdown of it inhibited KIRC cell proliferation, invasion, and migration [19]. The EMT process induced by nuclear factor- κ B signaling pathway may be the potential mechanism [19]. Small nucleolar RNA host gene 17 (SNHG17) was reported to accelerate cell proliferation and invasion in castration-resistant prostate cancer (CRPC) by targeting the miR-144/CD51 axis [20]. Furthermore, as lncRNAs that encode small nucleolar RNAs (snoRNAs), the prognostic value of SNHG17 and SNHG15 in KIRC patients has been investigated, and DNA hypomethylation might play a key role in elevated SNHG15 transcription in KIRC [21]. Recently, AC026356.2 and MELTF-AS1 were identified as immune-related lncRNA and showed a significant relationship with KIRC prognosis [22]. Zhang et al. established a lncRNA prognostic model based on LINC00460, MIAT, and LINC00443 from a competitive endogenous RNA regulatory network constructed in KIRC [23]. The function of HOTAIRM1 in KIRC was reported by Hamilton et al [24]. It suggested that HOTAIRM1 served a crucial role in kidney differentiation and suppressed angiogenic pathways induced by HIF1. Noteworthily, HOTAIRM1 is one of the few reported lncRNAs whose function is involved in the autophagy pathway. The degradation of PML-RARA oncoprotein and differentiation of myeloid cell is regulated by HOTAIRM1 via enhancing the autophagy pathway [25].

The fields of lncRNAs and autophagy are developing rapidly, and the regulatory role of lncRNA on autophagy in cancer also raised more attention in recent years. Many studies suggest that lncRNAs activate or inhibit autophagy by regulating autophagy-related genes and pathways, leading to promote or suppress tumor progression, depending on tumor microenvironment or cell environment pressure. Hence, there are four functional forms of cross regulation between lncRNAs and autophagy in cancer, lncRNAs promoting cancer by activating autophagy or inhibiting autophagy, lncRNAs suppressing cancer by activating autophagy or inhibiting autophagy [26]. In renal cancer, some autophagy-related lncRNAs have already been well studied. HOTAIR was reported to negatively target miR-17-5p to activate cell autophagy which mediated by Beclin1, resulting in sunitinib resistance in renal cancer cells [27]. In the study of Su et al. [28], 3-MA (an autophagy inhibitor) could reverse the inhibition of RCC cell proliferation, migration, and invasion induced by HOTTIP silencing. Further research found that the modification of HOTTIP affected RCC cell autophagy through the PI3K/Akt/Atg13 signaling pathway. In addition, RCC chemoresistance was known to be enhanced by lncRNA KIF9-AS1 which regulates autophagy signaling via miRNA-497-5p [29]. In summary, the role of autophagy regulated by lncRNAs in RCC is not only tumor progression, but also drug resistance.

In our current work, 17 autophagy-related lncRNAs were firstly identified in KIRC. The prognosis model was established, and the results indicated that the risk signature is a promising predictive indicator for patients with KIRC. Furthermore, to extract biological meaning from the identified differentially risk score, GSEA analysis was performed and immune-related GO terms were significantly enriched. These results declared that for high-risk patients, the immune response may be activated by the autophagy-related lncRNAs-miRNA-mRNA axis. Further studies should be performed to discover the miRNAs which connect the lncRNAs and mRNA to transmit signals and affect the immune response, transforming our identified autophagy-related lncRNAs into potential therapeutic targets for KIRC patients finally.

Conclusions

we successfully identified 17 autophagy-related lncRNAs and constructed a risk signature correlated with KIRC prognosis in the TCGA cohort. The results revealed that the signature is a potent and independent prognostic indicator for KIRC patients. Subsequent GSEA analysis suggested that the immune-related pathways were distinctly enriched in high-risk group. Further studies concentrating on our signature may disclose novel therapeutic targets for KIRC patients.

Abbreviations

Long non-coding RNAs

lncRNAs
The Cancer Genome Atlas
TCGA
Kidney renal clear cell carcinoma
KIRC
Receiver operating characteristic
ROC
Gene set enrichment analysis
GSEA
Renal cell carcinoma
RCC
Epithelial-to-mesenchymal transition
EMT
Human Autophagy Database
HADb
Hazard ratio
HR
Overall survival
OS
Gene Ontology
GO
Under the curve
AUC
Castration-resistant prostate cancer
CRPC
Small nucleolar RNAs
snoRNAs

Declarations

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Authors' contributions

YC, CM and CL designed the study. YC, XC and HD collected the data. TY, HB and JL analyzed the data. CM, SZ and YC sourced the literature. YC edited the manuscript. BL acquired the funding and supervised the whole study.

Competing Interests

The authors declare that they have no competing interests to disclose.

Availability of data and materials

All data generated or analyzed during this study were included in this published article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

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Figures

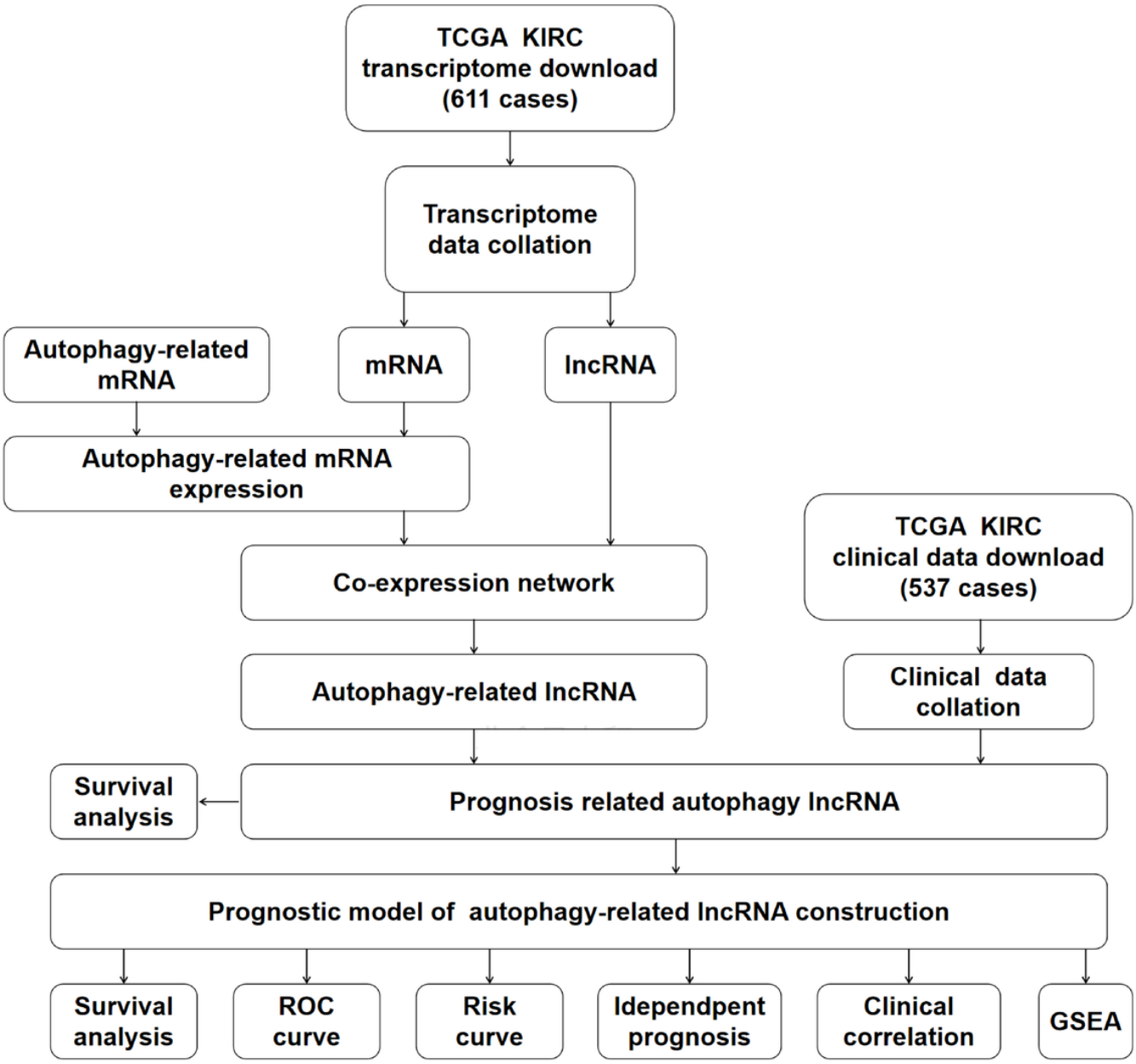


Figure 1

Main workflow for the study.

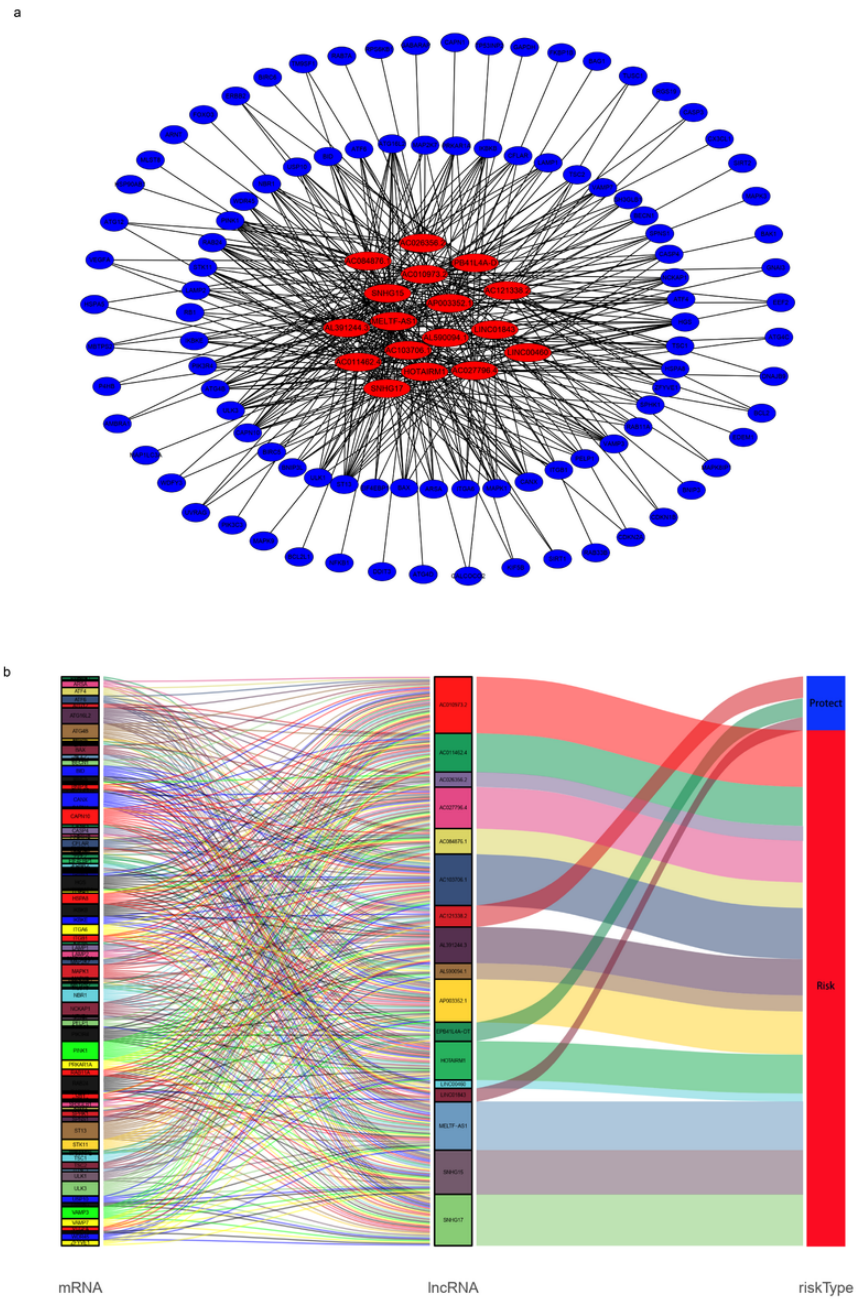


Figure 2

Network of lncRNAs with co-expression autophagy genes in KIRC. (a)The red ellipse indicates lncRNA. The blue ellipse indicates autophagy mRNA. The lines between them indicate the co-expression relationship. (b)Sankey diagram. (left column: autophagy-related mRNAs; middle column: the lncRNAs; right column: the risk type)

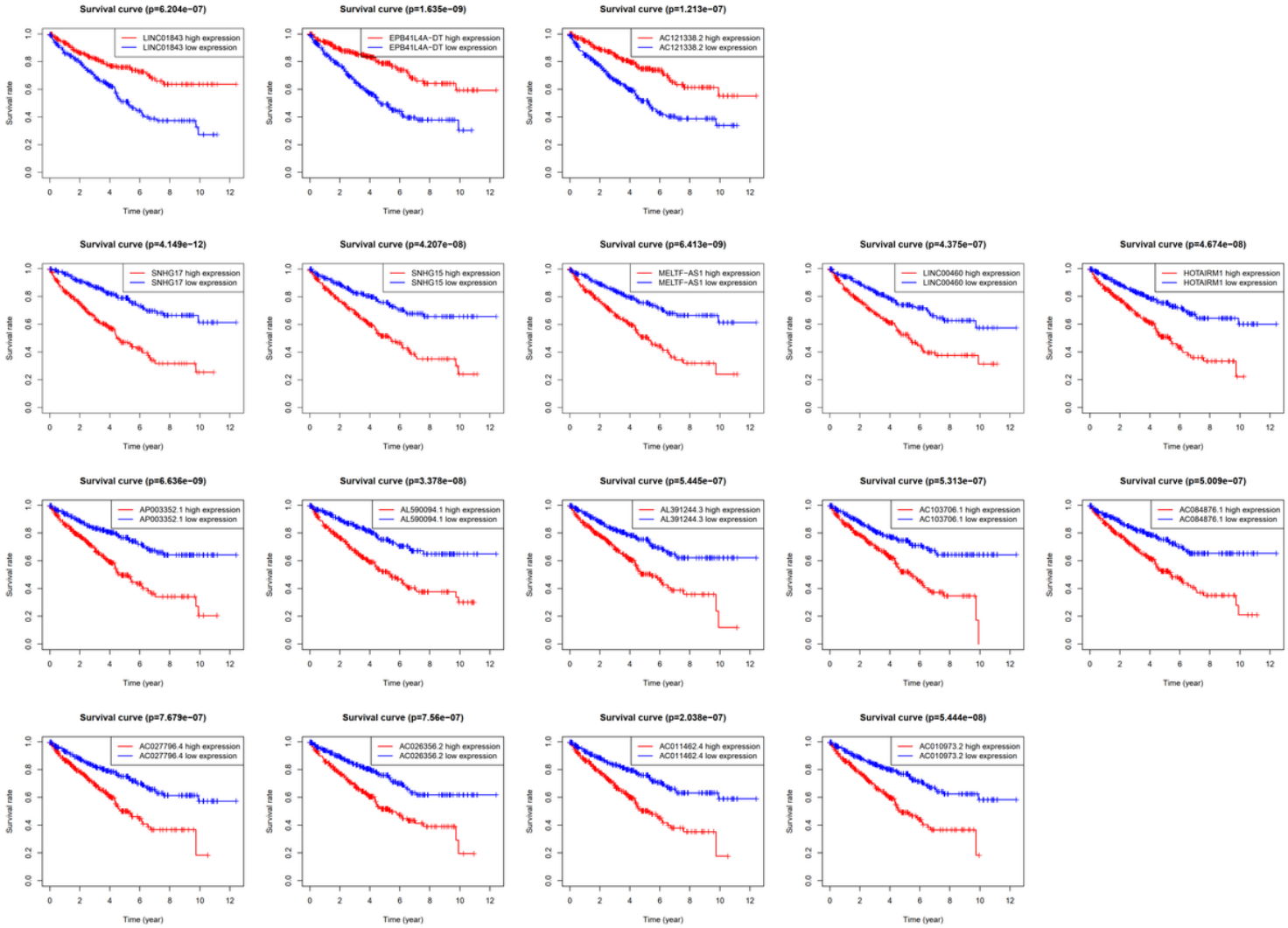


Figure 3

Survival analyses of 17 autophagy-related lncRNA. The top three survival curves are the survival analyses of protective lncRNAs, and higher expression predicts a higher survival rate. The curves under them are the survival analyses of risky lncRNAs for KIRC patients, and higher expression leads to shorter survival time.

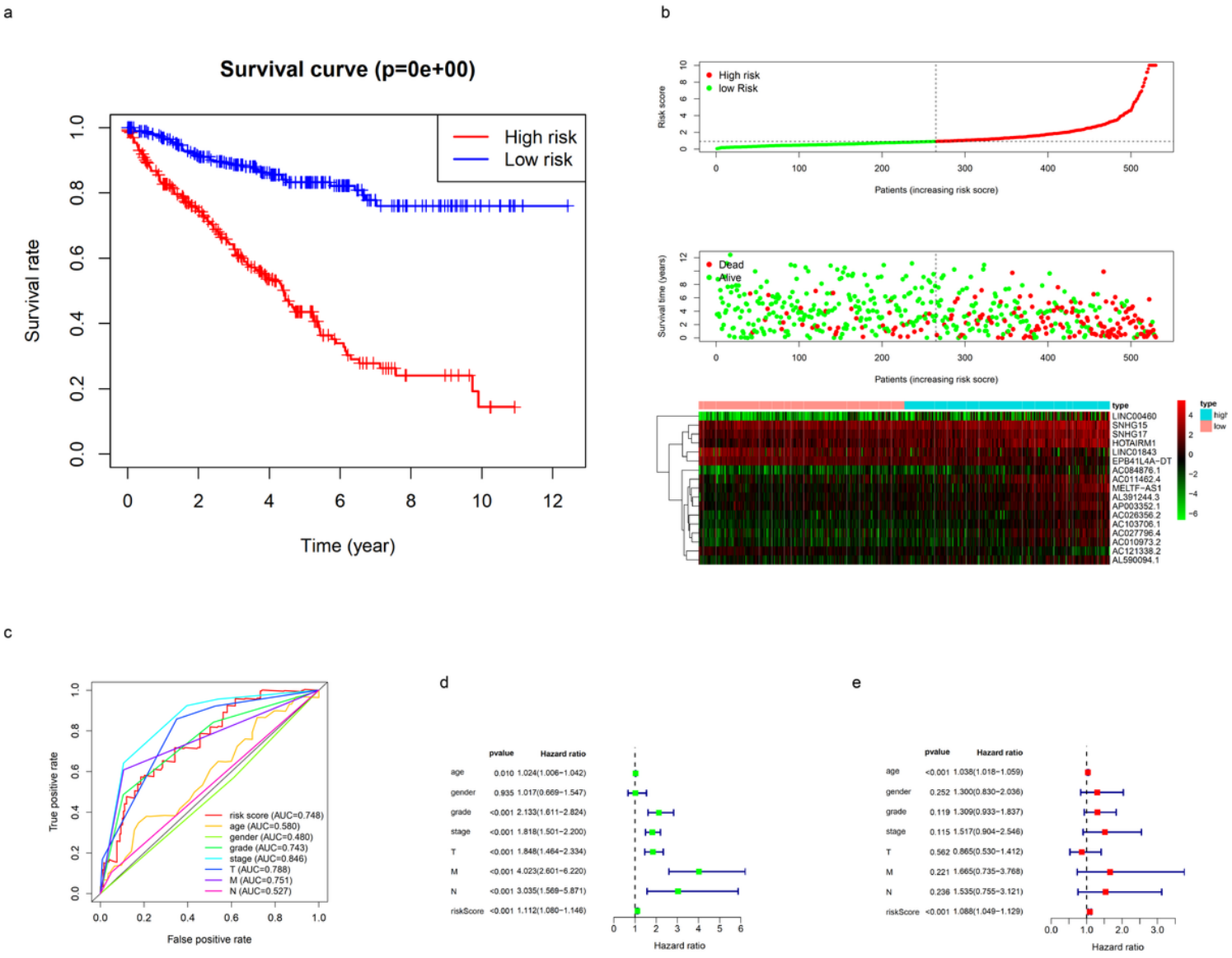
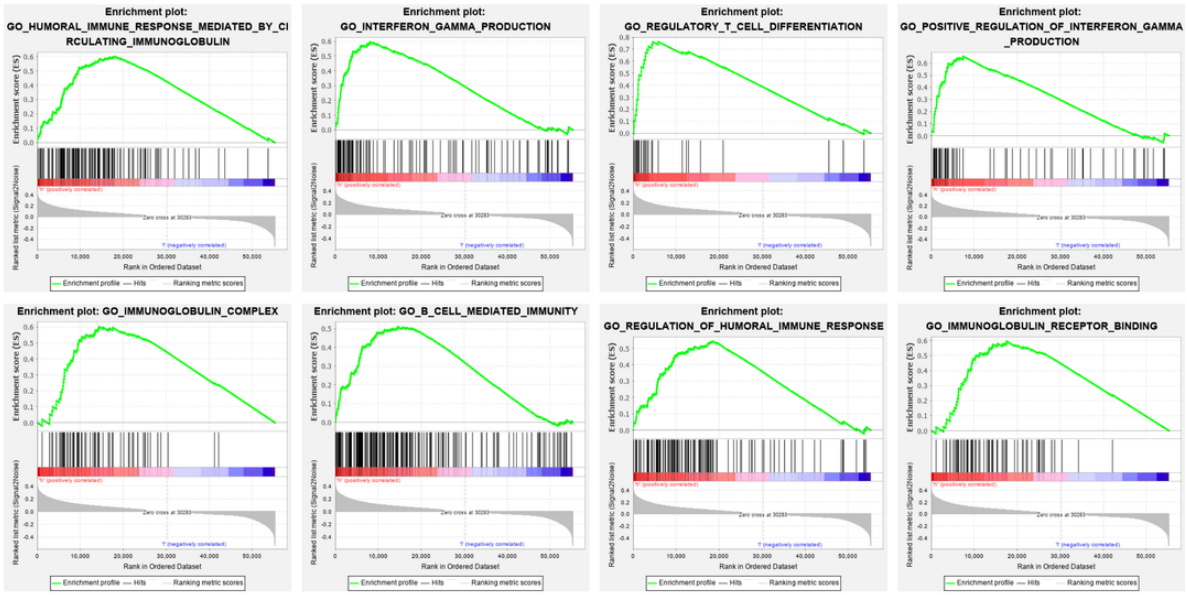


Figure 4

Prognosis model basing on risk signature. (a)The survival analysis relying on risk score. (b)Distributions of 17 lncRNA expression, survival status, and risk score for patients in high and low-risk groups. (c)The clinical variables dependent receiver operating characteristic (ROC) analyses. (d) Univariate Cox analysis. (e) Multivariate Cox analysis.

a



b

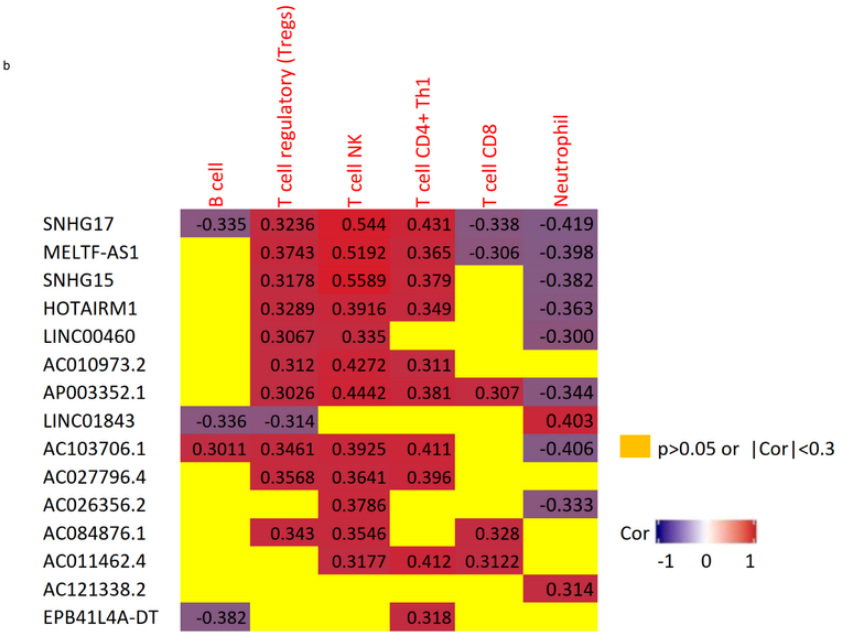


Figure 5

(a) Gene set enrichment analysis (GSEA) of risk signature. (b) The correlation between the expression of lncRNAs and immune cell infiltration level in KIRC. The yellow box indicates p value>0.05 or correlation coefficient (Cor) <0.3. The red box means positive correlation, the blue box means negative correlation, and the value in the box means the correlation coefficient.

Supplementary Files

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

- tables2.xlsx
- tableS1.xls