INTRODUCTION

Renal cell carcinoma (RCC) is one of the most lethal urological tumours, accounting for 2%-3% of malignancies in the United States. There are many kinds of histologic subtypes, among which clear cell RCC (ccRCC) constitutes 70% of RCC. While nephrectomy is curative method for ccRCC, approximately 30% of patients will relapse during the course of disease.

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Currently, the American Joint Committee (AJCC) staging system and the Fuhrman grading system have been universally acknowledged for cancer management clinically. However, clinicians cannot acquire accurate information to estimate recurrence-free survival (RFS) or overall survival so that providing personalized treatment for ccRCC patients from the TNM and grade classification. This could be ascribed to the biological heterogeneity of cancer, and therefore, molecular exploration may help clinicians precisely make treatment decisions for ccRCC patients according to risk classification via acquiring biomarkers for prediction of recurrence. Thus, it is urgent to explore new biomarkers for discriminating high-risk patients who may be inclined to have a higher probability of recurrence, thus offering personalized cancer treatment after surgery.

Clear cell RCC is a highly heterogeneous disease, resulting from complicated interaction between genetic and environmental factors. Analysing gene expression profiles of different cancer tissues or cells, with different tumour stages, may be helpful for identification of characteristic risk gene signature in cancer. Nowadays, many researchers have focused on the gene expression profiles of ccRCC and tried to illuminate the underlying mechanism of progression. However, few of them are used clinically. Therefore, identifying a more precise and practical risk gene model for predicting prognosis is urgently needed.

In the present study, we identified the differentially expressed genes between the normal kidney samples and ccRCC tumour tissues by gene expression microarray. A risk gene signature that can reflect the biological heterogeneity of different ccRCC patients and effectively predict clinical RFS was established via integrating gene expression profiles with matched clinical patient information. Moreover, we combined both genomic and clinical features of patients to construct a nomogram model for more accurate recurrence evaluation and facilitating personalized management of ccRCC patients after surgery.

2 | MATERIALS AND METHODS

2.1 | Study design and ccRCC specimen cohorts

We retrospectively analysed 215 paraffin-embedded tumour tissues from ccRCC patients treated at the Sir Run Run Shaw Hospital (Hangzhou, China) between January 2004 and December 2008. Besides, we obtained a total of 123 ccRCC patients with global gene expression profiling and detailed clinical information from TCGA database serving as external validation data set (Table 1). Computer-generated random numbers were applied to divide 215 specimens into a training cohort with a number of 107 samples and a validation cohort with a number of 108 samples. Total RNA was obtained from clinical FFPE samples by using the QIAGEN FFPE RNeasy kit (Qiagen GmbH). The quality of RNA was tested by NanoDrop 2000 spectrophotometer (ThermoFisher Scientific), and total RNA was amplified by Ovation FFPE WTA System (NuGEN).

| Variables                  | Training dataset (N = 107) | Testing dataset (N = 108) | External validation dataset (N = 123) |
|----------------------------|----------------------------|---------------------------|--------------------------------------|
| Gender                     | N% or mean (range)         |                           |                                      |
| Male                       | 73 (68.2)                  | 62 (57.4)                 | 68 (55.3)                            |
| Female                     | 34 (31.8)                  | 48 (42.6)                 | 55 (44.7)                            |
| Age (years)                |                            |                           |                                      |
| Male                       | 58.16 (36-79)              | 59.88 (37-83)             | 57.98 (37-79)                        |
| Female                     | 60.61 (39-82)              | 61.28 (33-86)             | 60.56 (37-82)                        |
| Tumor stage                |                            |                           |                                      |
| I                          | 47 (43.9)                  | 55 (50.9)                 | 50 (40.7)                            |
| II                         | 14 (13.1)                  | 12 (11.1)                 | 27 (22.0)                            |
| III                        | 26 (24.3)                  | 26 (24.1)                 | 28 (22.8)                            |
| IV                         | 20 (18.7)                  | 15 (13.9)                 | 18 (14.5)                            |
| Fuhrman grade              |                            |                           |                                      |
| I                          | 13 (12.1)                  | 3 (2.8)                   | 9 (7.2)                              |
| II                         | 57 (53.2)                  | 53 (49.1)                 | 67 (54.6)                            |
| III                        | 32 (29.9)                  | 39 (36.1)                 | 32 (26.0)                            |
| IV                         | 5 (4.7)                    | 13 (12.0)                 | 15 (12.2)                            |
| Lymph node invasion        | 12 (11.2)                  | 9 (8.3)                   | 11 (8.9)                             |
| Necrosis                   | 33 (30.8)                  | 44 (40.7)                 | 37 (30.1)                            |
| Number of events           | 48 (44.9)                  | 38 (35.2)                 | 58 (47.2)                            |

TABLE 1 Patient characteristics of three cohorts
were shown in Figure 1. These studies were conducted with approval from the Ethics Committee.

2.2 | Microarray data and differentially expressed gene analysis

cCRCC gene expression data (GSE68417) used in this study are available on GEO (https://www.ncbi.nlm.nih.gov/geo/). All raw data CEL files (Affymetrix Human Gene 1.0 ST Array) were processed under the same chip platform. These raw data files were downloaded and normalized by using a robust multi-array averaging method (expresso(data,bgcorrect.method = "rma", normalize.method="quantiles", pncorrect.method="pmonly", summary.method = "medianpolish"))⁹. A classical criterion of t test was adopted to identify DEGs with a change ≥ twofold, and P-value cut-off < .01 was considered to be statistically significant.

2.3 | Gene ontology analysis and Kyoto Encyclopedia of Genes and Genomes analysis

The Database for Annotation Visualization and Integrated Discovery (DAVID) was used to conduct the Gene ontology analysis (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG).¹⁰,¹¹ We used the human genome as the analysis background and defined P < .01 to be statistically significant.

2.4 | Identification and validation of the prognostic gene signature

In order to screen out the risk gene signature, R software (version 3.2.1) and the ‘glmnet’ package were applied to perform the LASSO Cox regression analysis in the training data set. The LASSO penalty was used to achieve shrinkage and variable selection simultaneously, and the optimal values of the penalty parameter lambda were determined through 10 times cross-validations. Genes which were significantly correlated with RFS in cCRCC were screen out based on the optimal lambda value. The risk score of each patient was calculated based on the expression level of each prognostic mRNA expression and its associated coefficient. Then, the patients in each data set were divided into low-risk and high-risk groups according to their mean risk score. Finally, we performed the Kaplan-Meier estimator and the log-rank test to assess RFS differences between the above low-risk and high-risk groups.

2.5 | Validation of hub gene expression via quantitative real-time PCR

The expression of identified hub genes was determined by qRT-PCR. Fifty normal kidney samples and 50 cCRCC tumour samples were obtained from Sir Run Run Shaw Hospital for validation of hub genes solely. The mRNA expression levels of risk genes were normalized to an internal standard (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). PCR primers were used as follows: DCN: forward, 5’-GACAAGGTCCGCCAGTTATG-3’; reverse, 5’-TCGTCT AGTCTTCACTATTCTG-3’; FGF2: forward, 5’-AGAAGAGGCGACCC TACATCA-3’; reverse, 5’-CGGTTAGCACACACTCTTGG-3’; STAT6: forward, 5’-GTGCTCGCCACTTGCCAATG-3’; reverse, 5’-TGGATCTCC ACTTACGTGTTG-3’; CD19: forward, 5’-GGCCCGAGGAACCTCTAGT-3’; reverse, 5’-TGATCTCGCCACTTGGCAAGT-3’; MAP4K1: forward, 5’-GTGCTGGGACCTGACATTTCGT-3’; reverse, 5’-CGTGTCTGGGACTCTGTGCT-3’;

2.6 | Statistical analysis

We did a multivariate Cox regression analysis using backward selection to testify the independent of different indicators; variables (P < .05) were remained in the final model for nomogram construction.

Our nomogram was generated via rms package in R platform and the multivariable Cox regression model. Comparisons between the nomogram and other prognostic systems were performed by using the rcorrp.cens package in Hmisc in R. Statistical analysis was done in R (version 3.2.1) and SPSS (version 22.0). P-value < .05 was deemed significant.
3 | RESULTS

3.1 | Identification of DEGs between normal kidney samples and ccRCC tumour samples

In the microarray analysis, with the criteria $P < .01$ and fold control (FC) ≥1.5, 580 genes were identified to be differentially expressed between 14 normal kidney samples and 29 ccRCC tumour samples. The volcano plot and heatmap were presented in Figure 2A and 2.

3.2 | Functional enrichment analysis of DEGs and selection of risk gene signature

Then, 580 DEGs were put into DAVID for functional analysis. The GO analysis, including molecular function, cellular component (CC) and biological process (BP), showed that these genes were primarily involved in cell adhesion, positive regulation of cell motility and WNT signalling pathway (Figure 2C). To further elucidate the potential functional pathways of DEGs, we conducted KEGG pathway enrichment analysis. B cell receptor signalling pathway, NF-kappa B signalling pathway and WNT signalling pathway were considered to be the most significantly enriched pathways (Figure 2D). LASSO Cox regression was used for further analysis, and 5 of these differentially expressed genes were identified to be significantly related to RFS of ccRCC (Table 2). The risk scores of each patient were calculated by a formula which was derived from the expression level of five risk genes weighted by regression coefficient.

3.3 | To further demonstrate the expression of risk genes via RT-QPCR

To further confirm the expression of identified risk genes from network-based analysis, RT-QPCR assay of five hub genes (CD19, FGF2,
MAP4K1, DCN and STAT6) were performed between 50 normal kidney samples and 50 ccRCC tumour samples. The mRNA expression levels of five hub genes were consistent with microarray results (Figure 3). In conclusion, these results indicated that these five risk genes (CD19, FGF2, MAP4K1, DCN and STAT6) were actually differentially expressed between normal kidney samples and ccRCC tumour samples.

### 3.4 Construction and validation of risk gene signature score model for predicting RFS of ccRCC patients

The 215 patients were randomly divided into training data set (n = 107) and testing data set (n = 108). Median follow-up was 61.4 months (IQR 87.7-23.1) for patients in the training data set and 37.1 months (IQR 51.7-25.2) for those in the testing data set. The patients in training data set were divided into low-risk group (n = 57) and high-risk group (n = 50) based on the mean risk score. Patients in the high-risk group indicated a worse clinical prognosis when compared with those in the low-risk group (Figure 4A). The efficacy of our five gene-signature for RFS prediction of ccRCC patients was further verified in testing data set and external validation data set. The same risk gene score-based classifier was used to classify patients in testing data set and external validation data set into the high-risk and the low-risk groups. Consistent with the results described above, patients in the high-risk group had a significantly shorter RFS (Figure 4B and 4).

### 3.5 Independence of 5-gene signature risk score model for RFS prediction from clinical features

To determine whether the prognostic value of five gene-signature was independent of patient clinical features, we performed the univariable and multivariate Cox regression analyses using RFS as the dependent variable and five gene-signature score, age, gender, tumour stage, grade, lymph node invasion and necrosis as covariates in

**TABLE 2** mRNA significantly associated with the recurrence-free survival in Training dataset. T/N: expression in ccRCC samples/expression in normal kidney samples

| Variables   | Expression(T/N) | HR     | P value  | Coefficient | Description                  |
|-------------|-----------------|--------|----------|-------------|------------------------------|
| STAT6       | Up-regulated    | 4.014  | <.0001   | 1.674       | Signal transducer and activator of transcription 6 |
| CD19        | Up-regulated    | 1.817  | <.0001   | 0.749       | CD19 molecule                |
| MAP4K1      | Up-regulated    | 1.802  | .004     | 0.714       | Mitogen-activated protein kinase kinase kinase 1 |
| FGF2        | Down-regulated | 1.674  | .005     | −0.643      | Fibroblast growth factor 2   |
| DCN         | Down-regulated | 1.798  | .007     | −0.756      | Decorin                      |

**FIGURE 3** To further confirm the expression of identified hub genes in clinical samples: CD19, FGF2, MAP4K1, DCN and STAT6
each data set. We found that the five gene-signature was significantly related to RFS of ccRCC patients after adjusting for clinical features in the training data set (HR = 2.107, CI = 1.689-2.773, P = .002), the testing data set (HR = 2.418, CI = 1.683-3.417, P < .0001) and the external validation data set (HR = 2.195, CI = 1.645-2.595, P < .0001) (Tables 3-5).

**TABLE 3** Univariable and multivariable Cox regression analysis of risk gene signature and other clinical features in training dataset

| Variables          | Univariate analysis | Multivariate analysis |
|--------------------|---------------------|-----------------------|
|                    | HR                  | 95% CI of HR          | P value | HR                  | 95% CI of HR          | P value |
| risk gene signature| 2.509               | 1.990-3.163           | <.0001  | 2.107               | 1.689-2.773           | .002    |
| Age                | 1.003               | 0.976-1.030           | .834    | 0.993               | 0.965-1.021           | .615    |
| Gender             | 1.023               | 0.572-1.831           | .938    | 1.065               | 0.559-2.028           | .849    |
| Tumour stage       | 2.475               | 1.826-3.354           | <.0001  | 1.490               | 1.056-2.007           | .019    |
| Fuhrman grade      | 3.091               | 1.979-4.826           | <.0001  | 1.713               | 1.016-2.887           | .031    |
| Necrosis           | 5.857               | 3.159-10.859          | <.0001  | 2.075               | 1.012-4.256           | .043    |
| Lymph node invasion| 16.233              | 7.369-35.761          | <.0001  | 2.972               | 1.176-7.510           | .021    |

**TABLE 4** Univariable and multivariable Cox regression analyses of risk gene signature and other clinical features in testing dataset

| Variables          | Univariate analysis | Multivariate analysis |
|--------------------|---------------------|-----------------------|
|                    | HR                  | 95% CI of HR          | P value | HR                  | 95% CI of HR          | P value |
| risk gene signature| 2.727               | 2.011-3.914           | <.0001  | 2.418               | 1.683-3.417           | <.0001  |
| Age                | 1.005               | 0.979-1.032           | .728    | 1.026               | 0.995-1.059           | .105    |
| Gender             | 1.008               | 0.514-1.975           | .981    | 0.782               | 0.352-2.140           | .782    |
| Tumour stage       | 2.262               | 1.795-3.524           | <.0001  | 2.228               | 1.442-3.442           | <.0001  |
| Fuhrman grade      | 2.642               | 1.732-4.031           | <.0001  | 2.153               | 1.245-3.724           | .006    |
| Necrosis           | 4.561               | 2.214-9.397           | <.0001  | 2.326               | 1.050-5.154           | .038    |
| Lymph node invasion| 5.327               | 2.403-11.809          | <.0001  | 3.495               | 1.400-8.723           | .007    |

Besides, we introduced the stratification based on tumour stage. We further stratified ccRCC patients into two subgroups where the AJCC stages I and II were fictitiously described as an early-stage stratum and the AJCC stages III and IV as a late-stage stratum. Result from Figure 5A-F indicated that the risk gene signature still had the ability to distinguish that the outcome of patients with high-risk
score was dramatically worse than that with low-risk score both in the early-stage and late-stage stratum.

Receiver operating characteristic analysis was also performed to testify the specificity and sensitivity of RFS prediction in each data set (Figure 6A-C). The risk gene signature score model possessed a similar predictive power compared with AJCC stage and tumour grade for the prognostic evaluation of ccRCC patients.

**TABLE 5** Univariable and multivariable Cox regression analyses of risk gene signature and other clinical features in external validation dataset

| Variables                  | Univariate analysis | Multivariate analysis |
|----------------------------|---------------------|-----------------------|
|                            | HR      | 95% CI of HR | P value | HR      | 95% CI of HR | P value |
| risk gene signature       | 2.922   | 2.570-3.354 | <.0001  | 2.195   | 1.645-2.595 | <.0001  |
| Age                       | 1.007   | 0.983-1.032 | .570    | 0.999   | 0.974-1.024 | .935    |
| Gender                    | 1.363   | 0.801-2.320 | .254    | 1.378   | 0.788-2.410 | .261    |
| Tumour stage              | 3.142   | 2.335-4.226 | <.0001  | 1.519   | 1.032-2.236 | .034    |
| Fuhrman grade             | 4.354   | 2.873-6.598 | <.0001  | 2.245   | 1.239-3.879 | .004    |
| Necrosis                  | 4.251   | 2.474-7.302 | <.0001  | 2.545   | 1.395-4.644 | .002    |
| Lymph node invasion       | 8.721   | 4.132-18.409| <.0001  | 2.791   | 1.253-6.219 | .012    |

**FIGURE 5** Using Kaplan-Meier survival analysis to testify the independence of our risk gene signature from AJCC stage. The patients from each data set were stratified into subgroups. The risk gene signature was applied to the low-stage patients (A, C, E) and high-stage patients (B, D, F)
Construction of nomogram combined 5-gene signature with the other clinical features for personalized prediction

To come up with a useful approach to predict the risk of recurrence so as to facilitate personalized management of ccRCC patients, we constructed a nomogram which combined our five gene-signature, and clinical features for predicting 3-year, 5-year and 10-year RFS (Figure 7A). Results from Figure 7B-D indicated that the line segments in the calibration plots were close to the 45° line which meant the well prediction, demonstrating that our nomogram was useful for prediction of 3-year, 5-year and 10-year RFS. Besides, the C-index of our nomogram was 0.859, 95% confidence interval (CI) 0.8149–0.9031, which was significantly higher (P < .01) than the SSIGN prognostic system (0.808), 95% CI 0.758–0.858. Besides, while we excluded the risk gene signature from the nomogram, the C-index of nomogram dropped to 0.819, 95% CI 0.775–0.863. These results indicated that our nomogram could serve as a predictor for RFS of ccRCC.

DISCUSSION

In this retrospective study, results indicated that our risk gene signature model developed in this research could categorize patients who had significantly different RFS into the low- and high-risk groups.
Besides, the efficacy of risk gene signature was verified in both internal and external validation cohorts, respectively. Importantly, a novel prognostic nomogram for precisely predicting RFS of ccRCC patients after nephrectomy was constructed based on the expression of risk gene signature and clinical risk features.

Cancers are recognized as heterogeneous disease. Thus, identifying the dysregulated genes in tumour carcinogenesis and progression could be helpful for improving prognostic and therapeutic strategies. Nowadays, development in microarray has contributed to the acquisition of large amounts of data which is useful for exploring molecular mechanisms, risk stratification and guiding strategies for clinical therapy in different cancers. In our study, microarray analysis was performed to acquire different expressed genes between normal kidney and ccRCC. Risk gene signature classifier was generated to predict recurrence risk, and its prognostic value was verified in both internal and external validation cohorts. Moreover, this indicator is independent of clinical features and possessed a similar predictive power compared with those widely used indicators for ccRCC such as AJCC stage and tumour grade. Among these genes, MAP4K1 was previously known to positively regulate cell motility and thereby to influence tumour cell invasion in the medulloblastoma and colon carcinoma. Beside, Lourdes and Wang, Y indicated that the MAP4K1 was related to the progression of bladder cancer, and STAT6 was found to promote intestinal tumorigenesis in the mouse model via inhibition of cytotoxic CD8 response and was involved in lymphoma. It is reported that activation of FGFR1 by its ligand fibroblast growth factor 2 (FGF2) could promote cell proliferation, epithelial-mesenchymal transition and invasion in lung cancer. Overexpression of FGF2 could also induce EMT in malignant pleural mesothelioma cells via MAPK/MMP1 signal and confer the poor prognosis. DCN is found to be a novel biomarker for the diagnosis of colon cancer by using iTRAQ-tagging and 2D-LC-MS/MS. Researchers found that T cells with chimeric antigen receptors (CAR T cells) which targets human CD19 (hCD19) have shown great efficacy against B cell malignancies. Therefore, our risk gene signature could potentially serve as a predictive appliance for personalized treatment and might also be potential target for clinical therapeutic targets of ccRCC.

Finally, a nomogram was constructed for prediction of an individual’s recurrence risk. Despite the fact that we, nowadays, often apply traditional indicators in clinic, such as tumour grade, stage and lymph node invasion (lymph node invasion owns highest hazard
ratios in our study), these factors are unable to guide personalized treatment.\textsuperscript{30,31} Importantly, when patients presented with the same stage or grade, these traditional factors are unable to predict an individual's risk. Therefore, our nomogram combined individual gene signature reflecting the biological heterogeneity of different ccRCC patients with traditional prognostic factors providing insights into a patient's clinicopathologic features so as to elevated the accuracy of individual RFS prediction.\textsuperscript{32,33} We also demonstrated the performance of our nomogram in validation cohorts. However, this research is retrospective and our sample size is still limited. Thus, our risk gene model and nomogram still require further validation in multicenter clinical trials. Besides, we will validate the efficiency of our nomogram in other ccRCC patient cohorts in the following studies.

5 | CONCLUSIONS

This is the first research to combine gene expression profiles with clinical information for predicting clinical prognosis of ccRCC patients. Our results show that the risk gene signature can effectively classify ccRCC patients into high and low-risk groups. Moreover, this nomogram might help clinicians accurately and personally predict the prognosis of patients with ccRCC after nephrectomy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

YLC, SJJ, LQX, JYL and GHL conceptualized the data; WH, YLC and SJJ contributed to methodology; LQX, LYL and DWX provided software; YLC, SJJ and ZYL investigated the study; LQX and LYL provided resources; YLC and SJJ curated the data; YLC wrote—original draft preparation; YLC wrote—review and editing; ZYL and LWX visualized the data; GHL supervised the study; GHL administrated the project; GHL acquired funding.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GEO (https://www.ncbi.nlm.nih.gov/geo/) and TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga).

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