Identification and validation of an eight-gene expression signature for predicting high Fuhrman grade renal cell carcinoma

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Clear cell renal cell carcinoma (ccRCC) is a malignancy with heterogeneous outcomes. Currently, renal mass biopsies are commonly employed to extract disease characteristics and aid prognosis. Although the pathological diagnosis of malignant disease is accurate in contemporary reports, the classification of Fuhrman grade using biopsy specimens remains far from promising. To generate a gene signature to distinguish high-grade ccRCC, we used the cancer genome atlas (TCGA) database to develop a gene expression signature for distinguishing high-grade (G3/4) from low-grade (G1/2) disease. The expression profile was further validated for performance and clinical use in 283 frozen renal cancer samples and 127 ex vivo renal mass biopsy samples, respectively. The area under curve (AUC) was used to quantify discriminative ability and was compared using the De-long test. Using the discovery dataset, we identified a 24-gene signature for high-grade disease with an AUC of 0.884. After applied to the development dataset, an eight-gene profile was defined and achieved an AUC of 0.823. Accuracy of eight-gene panel was maintained in the renal mass biopsies (RMB) samples (AUC = 0.821). In summary, using three-stage design, we validated an eight-gene expression signature for predicting high Fuhrman grade of ccRCC. This tool may help to reveal the characteristics of ccRCC biopsy specimens.

Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma (RCC).1 Approximately 80% of RCC cases are ccRCC and advanced ccRCC has poor prognosis.1 The widely used system for grading RCC is a nuclear grading system described in 1982 by Fuhrman.2 Increasing grade describes degrees of biologic aggressiveness and is associated with adverse outcomes. Fuhrman grade represents one of the most significant prognostic variables in patients with all stages of RCC.2,3 There were several researchers attempted to simplify the Fuhrman grading system to improve interobserver reproducibility.4 Further studies compared the simplified two-tiered Fuhrman grading systems with a four-tiered grading system and confirmed that the two-tiered grading systems are equally as valuable as the conventional four-tiered Fuhrman grading system based on accuracy criteria and in predicting cancer-specific mortality in ccRCC.5,6 Histological diagnosis and Fuhrman grade (FG) of RCC are commonly established by surgical resection of tumors.7

Key words: biomarker, clear cell renal cell carcinoma, Fuhrman grade, TCGA, renal mass biopsy

Abbreviations: AUA: American Urological Association; AUC: area under curve; BCR: biospecimen core resource; ccRCC: clear cell renal cell carcinoma; DEPC: diethylpyrocarbonate; EMT: epithelial–mesenchymal transition; EAU: European Association of Urology; FDR: false positive rate; FG: Fuhrman grade; FUSCC: Fudan University Shanghai Cancer Center; KIRC: kidney renal clear cell carcinoma; LASSO: least absolute shrinkage and selection operator; NSS: nephron spearing nephrectomy; qRT-PCR: quantitative reverse transcription-PCR; ROC: receiver operating characteristic; RCC: renal cell carcinoma; RMBs: renal mass biopsies; TCGA: The Cancer Genome Atlas

Additional Supporting Information may be found in the online version of this article.

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Grant sponsor: Jieping Medical Foundation grant; Grant number: 320.6750.1382; Grant sponsor: Shanghai Municipal Commission of Health and Family Planning grant; Grant number: 2014yzl0102; Grant sponsor: Dingwei Ye, National Nature Science Foundation of China; Grant number: 81370073; Grant sponsor: Shanghai Rising Star Program; Grant number: 16QA1401100; Grant sponsor: National Nature Science Foundation of China; Grant number: 81502192

DOI: 10.1002/ijc.30535

History: Received 24 Jan 2016; Accepted 16 Nov 2016; Online 22 Nov 2016

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Int. J. Cancer: 140, 1199–1208 (2017) © 2016 UICC
Alternatively, renal mass biopsies (RMBs) may be considered for small lesions in selected individuals and for advanced RCC to establish RCC diagnosis. Pathological features from RMBs may guide active surveillance strategies, cryosurgery and radiofrequency ablation in early RCC and first-line therapy in advanced RCC. However, sample error and tumor heterogeneity have contributed to the inaccuracy of RMBs. Although the biopsy failure rate was reduced by improved biopsy approaches, the precise grading of ccRCC remains difficult.

Recent studies have attempted to characterize the molecular basis of aggressiveness and clinical outcome of ccRCC. With the advances in various expression profilings and bioinformatic technologies, efforts are being made to identify molecular classifiers to refine the current grading method for ccRCC. These models were mostly based on the information of tumor grade and stage. However, precise pathological grading of RMB samples remains difficult, and the accuracy of predicting high grade with histological method was as low as 70% in most centers. There is a need of ccRCC gene profile from RMB specimens that would lead to more precise Fuhrman grading.

In our study, we demonstrated that gene expression profiling of RMB specimens could yield diagnostic information for predicting high Fuhrman grade. An eight-gene signature is a useful tool, which was identified from gene expression profiles and validated in RMB dataset, with a high sensitivity and specificity for Fuhrman grading. The value of this gene signature may be in the RMBs to initiate an active surveillance protocol for the elderly or medical comorbid patients. Also, the signature may have potentially academic interest in molecular understanding about Fuhrman grading system.

**Patients and Methods**

**Patient samples**

The Cancer Genome Atlas (TCGA) dataset, level 3 RNAseq expression data from kidney renal clear cell carcinoma (KIRC) samples by Illumina HiSeq 2000 RNA sequencing platform were obtained from TCGA data portal (https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/). Only tumor samples were taken into account in the study. Tumor transcriptomic profiles of 20,534 genes were measured in 478 primary ccRCC patients. Clinical information, including intact FG for selected subjects, was retrieved from the "Clinical Biotab" section of the data matrix based on the Biospecimen Core Resource (BCR) identification numbers of the patients. Extended demographic parameters for these patients, characterized by TCGA consortium, are shown in Table 1.

The Fudan University Shanghai Cancer Center (FUSCC) development set cohort consisted of 283 patients with histologically confirmed ccRCC by an experienced pathologist who had undergone nephron sparing nephrectomy (NSS) or nephrectomy at FUSCC without any pretreatment. These patients were consecutively enrolled from 2009 to 2012. The frozen tumor tissues were stored at −80°C once resected.

The RMB set was acquired from June 2013 to July 2014 at FUSCC for 127 ex vivo biopsy patients who underwent radical or partial nephrectomy for suspicious renal mass. Six core biopsies were taken from each tumor using an 18-gauge biopsy needle (two was obtained from a central location and four from a peripheral location) immediately after partial or radical nephrectomy as previously described to mimic RMBs. Three cores were separately snap-frozen in liquid nitrogen and stored at −80°C for RNA preparation. The other three remaining cores were fixed in 10% formalin for standard histological processing. Histological type and Fuhrman grade of biopsies specimens were reviewed by the same pathologist. Clinical data including pathological examination of surgical specimen were collected from electronic medical records at FUSCC. Our study was approved by the ethical committee of FUSCC, and each patient provided written informed consent before participation.

**RNA preparation and cDNA synthesis**

Each frozen tissue specimen was cut into small pieces using a clean disposable operation blade in RNase-free 1.5 ml Eppendorf tubes on ice. Then, 0.4 ml Trizol (Invitrogen, Life Technology, Carlsbad, CA) was added to the tubes and samples were ground manually using a grinding device (OSE-Y10/Y20, TIANGEN, Beijing, China) with a disposable RNase-free grinding rod (WL046, TIANGEN). Next 0.6 ml Trizol was added after grinding and tubes were briefly vortexed, and then 200 µl chloroform (Merck #102445) was added. Phase separation was performed manually without touching the lower phase and RNA precipitation was completed with 500 µl isopropanol. The RNA pellets were washed with 1 ml of 70% ice-cold ethanol, and the RNA was resolved with 50 µl diethylpyrocarbonate (DEPC) water and digested using 2U DNase at 37°C for 20 min followed by ethanol precipitation.

RNA extractions of core-biopsy samples were similar to that in uncut tissues. The total RNA of the three biopsy cores was mixed together before treatment with DNase. cDNA preparation was performed according to manufacturer's
instructions using a RevertAid First Strand cDNA Synthesis Kit (K1622; Thermo Fisher Scientific, Waltham, MA). Then, 500 ng template RNA was used in the reverse transcription reaction with 1 μl of random primer (0.2 μg/μl) in a total volume of 20 μl. The reaction mixture was incubated for 5 min at 25°C followed by 60 min at 42°C. cDNA concentration was measured using a NanoVue (28923215, GE Healthcare), and cDNA was diluted to 200 ng/μl with DEPC water.

Table 1. Clinicopathological characteristics of patients with ccRCC in the TCGA and FUSCC development cohorts

| Variable       | TCGA cohort |          |          | FUSCC development cohort |          |
|----------------|-------------|----------|----------|--------------------------|----------|
|                | Overall     | Low grade | High grade | Overall                  | Low grade |
|                | (n = 478)   | (n = 215) | (n = 263) | (n = 283)                | (n = 134) |
|                | Age (year)  |          |          |                          |          |
|                | Median (range) | 61 (26–90) | 60 (26–90) | 62 (32–90)              | 56 (17–86) |
|                | Gender      |          |          |                          |          |
|                | Male        | 312 (65.3) | 123 (57.2) | 189 (71.9)              | 194 (68.6) |
|                | Female      | 166 (34.7) | 92 (42.8)  | 74 (28.1)               | 89 (31.4)  |
|                | pT          |          |          |                          |          |
|                | T1          | 235 (49.2) | 151 (70.2) | 84 (31.9)               | 197 (69.6) |
|                | T2          | 59 (12.3)  | 24 (11.2)  | 35 (13.3)               | 37 (13.1)  |
|                | T3          | 174 (36.4) | 40 (18.6)  | 134 (51.0)              | 39 (13.8)  |
|                | T4          | 10 (2.1)   | 0 (0.0)    | 10 (3.8)                | 10 (3.5)   |
|                | pN          |          |          |                          |          |
|                | N0          | 224 (46.9) | 104 (48.4) | 120 (45.6)              | 271 (95.8) |
|                | N1          | 16 (3.3)   | 3 (1.4)    | 13 (5.0)                | 6 (2.1)    |
|                | Nx          | 238 (49.8) | 108 (50.2) | 130 (49.4)              | 6 (2.1)    |
|                | pM          |          |          |                          |          |
|                | M0          | 401 (83.9) | 205 (95.3) | 196 (74.5)              | 272 (96.1) |
|                | M1          | 77 (16.1)  | 10 (4.7)   | 67 (25.6)               | 11 (3.9)   |
|                | Pathological stage |          |          |                          |          |
|                | Stage I     | 230 (48.1) | 150 (69.8) | 80 (30.4)               | 194 (68.6) |
|                | Stage II    | 48 (10.1)  | 21 (9.8)   | 27 (10.3)               | 34 (12.0)  |
|                | Stage III   | 122 (25.5) | 34 (15.8)  | 88 (33.5)               | 39 (13.8)  |
|                | Stage IV    | 78 (16.3)  | 10 (4.6)   | 68 (25.8)               | 16 (5.6)   |
|                | Fuhrman grade |          |          |                          |          |
|                | 1           | 9 (1.9)    | 9 (4.2)    | 0 (0.0)                 | 13 (4.6)  |
|                | 2           | 206 (43.1) | 206 (95.8) | 0 (0.0)                 | 121 (42.8) |
|                | 3           | 190 (39.7) | 0 (0.0)    | 190 (72.2)              | 122 (43.1) |
|                | 4           | 73 (15.3)  | 0 (0.0)    | 73 (27.8)               | 27 (9.5)   |
|                | Tumor size  |          |          |                          |          |
|                | Mean(SD)    | 1.684 (0.663) | 1.525 (0.523) | 1.811 (0.733)              | 5.08 (2.706) |
|                | Surgery     |          |          |                          |          |
|                | Radical nephrectomy | NA | NA | NA | 233 (82.3) |
|                | NSS         |          |          |                          |          |
|                |              | 50 (17.6) | 19 (14.2) | 31 (20.8) |
|                | Tumor necrosis |          |          |                          |          |
|                |              | 7.89 | 5.11 | 10.00 | <0.001 |

1χ² test or indicated otherwise.
2t test.
3Fuhrman grade distribution was examined by Chi-square test.
4Tumor necrosis indicates mean percentage of tumor necrosis.

Abbreviations: TCGA, the cancer genome atlas; FUSCC, Fudan University Shanghai Cancer Center; RMB, renal mass biopsy; low grade, G1 and G2; high grade, G3 and G4; NA, not available; NSS, nephron sparing surgery.
Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

qRT-PCR primer sequences of 24 candidate genes are listed in Supporting Information Table S1. All primers were used at an annealing temperature of 60°C. Then 200 ng cDNA template was applied for the SYBR Green (638320, Takara, Japan) qRT-PCR analysis per well. The amplification was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies), and all measurements were taken in triplicate. The melting curves of each measurement were checked; only the coordinate results were included in the subsequent analysis. ACTB (β-actin) was used as the internal control. The mean Ct value of each gene minus the mean Ct value of ACTB was calculated as ΔCt. The –ΔCt value of each gene was applied for binary logistic regression and model construction.

Data analysis

All the statistical analysis steps, including data preprocessing, gene selection, classification model construction and independent testing were performed with R software and packages from the Bioconductor project.22,23 Significant gene selection was performed by the method of least absolute shrinkage and selection operator (LASSO) using the LARS package.24 As a typical penalized regression method, LASSO selects predictive genes and simultaneously estimates the regression coefficients in the multiple linear regression model, and thus is particularly useful for candidate genes’ selection and parameter estimation in high-dimensional genomic data. The prediction accuracy was estimated by tenfold cross-validation, which means that the dataset was divided into ten approximately equally sized subsets; a prediction model was trained for nine subsets and prediction was conducted for the remaining subset. This training and testing process was repeated 10 times to include predictions for each subset. For the data obtained by qRT-PCR, the Mann–Whitney unpaired test was used for the comparison between low- and high-grade samples. All significance tests were two-sided, and a p value of <0.05 was considered significant. A stepwise logistic regression model was used to select predictive markers based on the development dataset. The predicted probability of being diagnosed with low- and high-grade tumors was used as a surrogate marker to construct a receiver operating characteristic (ROC) curve. Area under the ROC curve was used as an accuracy index to identify the best combination of multiple markers.

Results

Detailed workflow of our study is shown in Figure 1. Clinico-pathological characteristics of the TCGA and FUSCC development cohort cohorts are listed in Table 1. The tumor grade distributions of the TCGA and FUSCC development cohort were similar (p = 0.526). A total of 127 ex vivo biopsy patients were enrolled in the RMB cohort, including six benign and nine non-ccRCC cases as well as 112 ccRCC patients (Table 2).

TCGA data analysis and candidate gene selection

The RNAseq dataset included gene expression levels for 20,534 genes in 221 low FG and 269 high FG patients. First we performed LASSO analysis to investigate candidate genes that are related to FG. With the tenfold cross-validation process, a list of 87 candidate genes was identified. Second, in multivariate logistic regression, 24 genes remained statistically significant and were selected to build a logistic regression model. The diagnostic performance for the 24-gene logit model, measured by AUC, reached 0.88 (detailed in Table 3). Among which, nine genes were overexpressed in low-grade patients and 15 genes were overexpressed in high-grade patients.

Development of the expression signature in the FUSCC cohort

The 24 genes selected in the discovery set were further evaluated by qRT-PCR analysis in 283 Chinese ccRCC patients. In patients in TCGA cohort were mostly Caucasians and the development and RMB cohorts were all Asians. Sex distribution differences were not shown in both the development and RMB cohorts. High grade was correlated with higher percentages of tumor necrosis, both in TCGA and FUSCC development cohorts. High grade was correlated with higher percentages of tumor necrosis, both in TCGA and FUSCC development cohorts. High grade was correlated with higher percentages of tumor necrosis, both in TCGA and FUSCC development cohorts. High grade was correlated with higher percentages of tumor necrosis, both in TCGA and FUSCC development cohorts.
univariate analysis, 10 out of 24 genes (ATOH8, ATP1A3, C10orf4, CHMP4C, CNGA1, NCRNA00116, PLA2G15, PPPIR1A, SPOCK1 and TXNDC16) were confirmed to be significantly differentially expressed between low and high FG patients. In multivariate analysis, C10orf4 and TXNDC16 failed to reach statistical significance and thus were excluded from the final panel (Table 4).

The predicted probability of being diagnosed with high FG tumors from the logit model based on the eight genes was used to construct the ROC curve, as follows: logit\( p_{\text{high FG}} = 1.482 - 0.31 \times \text{CNGA1} + 0.09 \times \text{PPPIR1A} - 0.163 \times \text{ATOH8} + 0.147 \times \text{SPOCK1} + 0.107 \times \text{NCRNA00116} + 0.186 \times \text{PLA2G15} - 0.095 \times \text{CHMP4C} + 0.134 \times \text{ATP1A3}. \) The best cutoff point of this model is 0.3672. Possibility above 0.3672 suggested high grade ccRCC. The AUC for the established eight-gene expression signature was 0.823 (95% CI, 0.776–0.870; Fig. 2a), significantly higher than conventional clinical parameter such as tumor size (AUC = 0.648, 95% CI, 0.584–0.712).

### Table 2. Clinicopathological characteristics of patients in FUSCC RMB cohorts

| Variable | Overall (n = 127) | Benign (n = 6) | ccRCC (n = 112) | non-ccRCC (n = 9) | \( p ^ 1 \) |
|----------|------------------|--------------|-----------------|-----------------|--------|
| Age (year) | <0.05 | | | | |
| Median (range) | 53 (25–81) | 40.5 (36–53) | 54 (25–81) | 54 (31–68) | |
| Gender | 0.258 | | | | |
| Male | 87 (68.5) | 3 (50%) | 76 (67.9) | 8 (88.9) | |
| Female | 40 (31.5) | 3 (50%) | 36 (32.1) | 1 (11.1) | |
| \( pT \) | 0.430 | | | | |
| T1 | 88 (72.7) | – | 81 (72.3) | 8 (88.9) | |
| T2 | 15 (12.4) | – | 14 (12.5) | 1 (11.1) | |
| T3 | 17 (14.0) | – | 17 (15.2) | 0 (0.0) | |
| \( pN \) | 0.719 | | | | |
| N0 | 106 (87.6) | – | 98 (87.5) | 8 (88.9) | |
| N1 | 6 (5.0) | – | 6 (5.4) | 0 (0.0) | |
| Nx | 9 (7.4) | – | 8 (7.1) | 1 (11.1) | |
| \( pM \) | 0.811 | | | | |
| M0 | 116 (95.9) | – | 107 (95.5) | 9 (100.0) | |
| M1 | 4 (3.3) | – | 4 (3.6) | 0 (0.0) | |
| Grade | 0.486 | | | | |
| 1,2 | 73 (60.3) | – | 67 (59.8) | 6 (66.7) | |
| 3,4 | 48 (39.7) | – | 45 (40.2) | 3 (33.3) | |
| Pathological stage | 0.014 | | | | |
| Stage I | 89 (73.6) | – | 81 (72.3) | 8 (88.9) | |
| Stage II | 11 (9.1) | – | 10 (8.9) | 1 (11.1) | |
| Stage III | 16 (13.2) | – | 16 (14.3) | 0 (0.0) | |
| Stage IV | 4 (3.3) | – | 4 (3.6) | 0 (0.0) | |

\(^1 \chi^2\) test or indicated otherwise.
\(^2\) t test.

Abbreviations: FUSCC, Fudan University Shanghai Cancer Center; RMB, renal mass biopsy; ccRCC, clear cell renal cell carcinoma.

### Predictive capability of the eight-gene expression signature in RMB dataset

In molecular profile analysis, parameters estimated from the FUSCC development set were used to predict the probability of being diagnosed with high FG tumor for these patients. The AUC of the eight-gene expression signature was 0.821 (95% CI, 0.737–0.887; Fig. 2b). The eight-gene signature could distinguish grade IV ccRCC with an AUC of 0.925 (95% CI, 0.860–0.966; Fig. 2c) and it could identify malignant tumor from benign mass with an AUC of 0.915 (95% CI, 0.852–0.957; Fig. 2d). Moreover, this gene set had a good performance in mixed histology types (AUC = 0.818, 95% CI, 0.736–0.882; Fig. 2e).

By standard histological methods, ex vivo core biopsies of the 127 renal tumors yielded noninformative results in 8 cases and 28 misclassified cases with an AUC of 0.626 (95% CI, 0.531–0.719), significant lower than eight-gene signature (\( p = 0.001 \)). Using conventional clinical parameters (tumor size, age, gender, stage, necrosis) to predict high grade, AUC
was only 0.767 (95% CI, 0.676–0.842). After integrated the clinical parameter model with eight-gene signature, the AUC significantly increase to 0.904 (95% CI, 0.833–0.952, \(p = 0.0026\); Fig. 1f).

Table 3. Composition of the 24-gene signature based on TCGA database developing group (\(n = 478\)) multivariate analysis

| Gene     | Description                                                                 | Cytoband | UniGene | OR               | 95% CI             | \(p\) values |
|----------|------------------------------------------------------------------------------|----------|---------|------------------|--------------------|--------------|
| ATOH8    | Atonal homolog 8(Drosophila)                                                 | 2p11.2   | Hs.135569 | 0.744            | (0.602–0.920)      | 6.26E-03     |
| ATP1A3   | ATP1A3 ATPase, Na+/K+ transporting, alpha 3 polypeptide                      | 19q13.31 | Hs.515427 | 1.182            | (1.006–1.388)      | 4.22E-02     |
| C10orf4  | Fragile site, folic acid type, rare, fra(10)(q23.3) or fra(10)(q24.2) candidate 1 | 10q23.33 | Hs.586650 | 0.498            | (0.298–0.832)      | 7.72E-03     |
| C17orf79 | Coordinator of PRMT5, differentiation stimulator                             | 17q11.2  | Hs.462729 | 2.201            | (1.066–4.545)      | 3.29E-02     |
| CHMP4C   | Charged multivesicular body protein 4C                                       | 8q21.13  | Hs.183861 | 0.585            | (0.382–0.895)      | 1.35E-02     |
| CNGA1    | Cyclic nucleotide gated channel alpha 1                                      | 4p12     | Hs.1323  | 0.754            | (0.635–0.895)      | 1.24E-03     |
| EDA      | Ectodysplasin A                                                             | Xq12-q13.1 | Hs.105407 | 0.518            | (0.364–0.738)      | 2.73E-04     |
| FBXL3    | F-box and leucine-rich repeat protein 3                                      | 13q22    | Hs.508284 | 6.864            | (3.044–15.481)     | 3.45E-06     |
| GMDS     | GDP-mannose 4,6-dehydratase                                                  | 6p25     | Hs.144496 | 2.189            | (1.339–3.578)      | 1.78E-03     |
| ISL2     | ISL LIM homeobox 2                                                           | 15q23    | Hs.444677 | 2.041            | (1.411–2.952)      | 1.53E-04     |
| KISS1    | KISS-1 metastasis-suppressor                                                 | 1q32     | Hs.95008  | 1.275            | (1.056–1.54)       | 1.15E-02     |
| KLF2     | Kruppel-like factor 2                                                        | 19p13.11 | Hs.744182 | 0.725            | (0.533–0.986)      | 4.04E-02     |
| MYADML2  | Myeloid-associated differentiation marker-like 2                             | 17q25.3  | Hs.729645 | 2.172            | (1.237–3.816)      | 6.95E-03     |
| NCRNA00116 | Long intergenic non-protein coding RNA 116                                   | 2q13     | Hs.128499 | 1.981            | (1.246–3.148)      | 3.84E-03     |
| OAZ1     | Ornithine decarboxylase antizyme 1                                           | 19p13.3  | Hs.444627 | 0.092            | (0.037–0.229)      | 3.03E-07     |
| ODZ3     | Teneurin transmembrane protein 3                                             | 4q35.1   | Hs.130438 | 1.174            | (1.027–1.343)      | 1.91E-02     |
| PLA2G15  | Phospholipase A2, group XV                                                   | 16q22.1  | Hs.632199 | 2.715            | (1.504–4.901)      | 9.18E-04     |
| PPP1R1A  | Protein phosphatase 1, regulatory (inhibitor) subunit 1A                     | 12q13.2  | Hs.505662 | 0.858            | (0.779–0.947)      | 2.23E-03     |
| RAB40A   | RAB40A, member RAS oncogene family                                           | Xq22.1   | Hs.706904 | 1.903            | (1.369–2.645)      | 1.30E-04     |
| RAS1     | Related RAS viral (r-ras) oncogene homolog                                  | 19q13.33 | Hs.515536 | 2.117            | (1.300–3.447)      | 2.58E-03     |
| SPOCK1   | Sparc/osteonectin, ccwv and kazal-like domains proteoglycan (testican) 1     | 5q31.2   | Hs.596136 | 1.203            | (1.044–1.388)      | 1.08E-02     |
| SQSTM1   | Sequestosome 1                                                               | 5q35     | Hs.724025 | 3.194            | (1.684–6.057)      | 3.77E-04     |
| TXNDC16  | Thioredoxin domain containing 16                                             | 14q22.1  | Hs.532609 | 2.488            | (1.412–4.385)      | 1.61E-03     |
| VAMP3    | Vesicle-associated membrane protein 3                                       | 1p36.23  | Hs.66708  | 0.172            | (0.067–0.442)      | 2.50E-04     |

Abbreviations: TCGA, cancer genome atlas; CI, confidence intervals.

Performance of the eight-gene signature in outcome prediction

We applied the eight-gene signature equation to calculate the possibility of high FG in FUSCC development cohort. Then we trichotomized them into three groups. Kaplan-Meier curve was plotted (Supporting Information Fig. S1). We also evaluated the outcome predictive capability of our model in 478 TCGA patients’ overall survival. We found that patient with a high score of our model is associated with poor prognosis. In detail, the C-index of eight-gene model (eight-gene signature and stage) can reach 0.747. Adding eight-gene signature alone into SSIGN model, \(25\) C-index increased to 0.755 (C-index of SSIGN alone is 0.751, \(p = 0.048\)). We used bootstrapping to internally validate and calibrate three models.

Supporting Information Fig. S2 depicted the three models’ calibration curve, which plotted the predicted vs. observed 5-year survival of TCGA cohort. The plots demonstrated good calibration (R code in Supporting Information).

Discussion

In addition to patient characteristics and expertise of surgeons, the choice of treatment for ccRCC is mainly based on evaluating its biological potential. Hence, clear identification of prognostic factors like FG, tumor size would help urologists distinguish progressive malignancies that require immediate intervention or target therapies and indolent ones for which suitable for active surveillance or ablation. In parallel with the established imaging technology to determine T stage, grading of ccRCC is another important parameter for clinicians. However, accurate determination of FG is difficult because of the heterogeneity of RCCs and issues associated with incomplete sampling. In our study, a pragmatic approach was used to identify candidate biomarkers predicting high FG for this important
clinical need. With authoritative and high-dimensional genomic data of TCGA, we used the solid bioinformatics algorithm to develop the predictive gene panel. Moreover, we validated this gene panel using a RMB cohort. In the ex vivo RMB dataset, the accuracy of this eight-gene signature was significantly higher than conventional methods. Interestingly, the model performed well to distinguish malignant and grade IV tumors and non-ccRCC specimen did not affect much on this model. These make the model more valuable in clinical RMB practice. To our knowledge, this is the first gene expression signature for Fuhrman grade classification in ccRCC.

Renal biopsies are commonly performed on patients with late stage renal masses or small lesions followed by cryotherapy or ablation. The European Association of Urology (EAU) recommends biopsy for all patients undergoing active surveillance, and American Urological Association (AUA) lists RMB as an option for such patients. The accuracy of RMB has been demonstrated to be higher than 60% for histologic diagnosis (cancer vs. benign), however, it remains poor for grade. Previous studies evaluating RMB performance in tumor grading by histologic procedure suggested that the accurate diagnosis rate was nearly 60%, ranging from 43 to 75%. In our study, the eight-gene signature markedly increased the accuracy of tumor grading than histological method with an elevated AUC of 0.195 (p < 0.001). Also this gene panel had better discriminable ability than conventional parameters using tumor size, AJCC stage and necrosis. Therefore, this signature could increase the accuracy of histological diagnosis in RMB samples.

A major strength of our study is that the samples were derived from two large populations with high statistic power and low false positive rate (FDR). Another advantage of our study is that the 24-gene signature derived from the data of TCGA. That is because the quality of the clinical samples is quite important to obtain reliable and reproducible data of molecular profiling. Third, the following development and validation stages were qRT-PCR based which is a fast, quantitative method with high reproducibility and is widely applicable in hospitals. However, there are different results between RNAseq and qRT-PCR in TCGA discovery dataset and FUSCC development dataset. Since the purpose of our study is to make an easy to use method for predicting high-grade ccRCC, the final model is qRT-PCR based. To make

| Genes | OR (95% CI) | p | OR (95% CI) | p |
|-------|-------------|---|-------------|---|
| ATOH8 | 0.905 (0.857–0.956) | <0.001 | 0.851 (0.779–0.930) | <0.001 |
| ATP1A3 | 1.137 (1.058–1.221) | <0.001 | 1.142 (1.044–1.249) | 0.004 |
| C10orf4 | 0.925 (0.858–0.996) | 0.038 | 1.008 (0.885–1.149) | 0.903 |
| C17orf79 | 0.995 (0.946–1.046) | 0.836 | |
| CHMP4C | 0.923 (0.868–0.982) | 0.012 | 0.909 (0.889–0.999) | 0.041 |
| CNGA1 | 0.858 (0.795–0.926) | <0.001 | 0.733 (0.634–0.848) | <0.001 |
| EDA | 0.961 (0.904–1.021) | 0.199 | |
| FBXL3 | 1.018 (0.965–1.073) | 0.516 | |
| GMDS | 0.980 (0.933–1.029) | 0.414 | |
| ISL2 | 1.019 (0.964–1.078) | 0.509 | |
| KISS1 | 0.989 (0.952–1.026) | 0.546 | |
| KLF2 | 0.957 (0.874–1.048) | 0.343 | |
| MYADML2 | 0.985 (0.956–1.015) | 0.330 | |
| NCRNA00116 | 1.056 (1.002–1.113) | 0.044 | 1.112 (1.023–1.209) | 0.013 |
| OAZ1 | 0.976 (0.899–1.060) | 0.573 | |
| ODZ3 | 1.032 (0.972–1.097) | 0.306 | |
| PLA2G15 | 1.121 (1.033–1.215) | 0.006 | 1.206 (1.065–1.367) | 0.003 |
| PPP1R1A | 1.095 (1.034–1.160) | 0.002 | 1.094 (1.007–1.187) | 0.033 |
| RAB40A | 0.993 (0.942–1.047) | 0.801 | |
| RRA5 | 1.022 (0.978–1.069) | 0.330 | |
| SPOCK1 | 1.107 (1.047–1.170) | <0.001 | 1.16 (1.078–1.248) | <0.001 |
| SQSTM1 | 1.015 (0.963–1.070) | 0.583 | |
| TXNDC16 | 0.907 (0.841–0.979) | 0.012 | 0.99 (0.867–1.132) | 0.888 |
| VAMP3 | 1.005 (0.969–1.042) | 0.786 | |

Abbreviations: FUSCC, Fudan University Shanghai Cancer Center; CI, confidence intervals.
Figure 2. Receiver operating characteristic (ROC) curve analysis of the eight-gene signature in the development and RMB set. In order to compare the predictive value of the eight-gene signature, we analyzed the ROC curve the signature in different datasets. ROC plots for the eight-gene panel discriminating high Fuhrman grade in the (a) FUSCC development set and (b) all ccRCC in ex vivo RMB set and (c) ex vivo RMB set, only grade IV ccRCC included, (d) malignant tumor vs. benign renal mass in ex vivo RMB set, (e) high grade (grade 3,4) in both ccRCC and non-ccRCC in ex vivo RMB set. (f) Comparison of AUC in four models. RMB represent standard histological method. Clinical parameters include age, gender, tumor size, AJCC stage and necrosis. Overall model was obtained by multivariate regression analysis for the combination of clinical parameter and eight-gene signature. AUC, area under the curve. [Color figure can be viewed at wileyonlinelibrary.com]
test the reproducibility of this model, we validate our results in biopsy samples with qRT-PCR method.

Along with the development of medical technology and molecular therapy, the treatment methods of ccRCC improved. Less invasive strategies such as ablative therapy or active surveillance are now being discussed for the treatment of small ccRCCs. Patient selection and cancer subtype classification is becoming important. So far, many approaches have been applied to establish the molecular subtypes of cancers. These attempts made a big step forward in the understanding of ccRCC. However, in these models, FG and stage remained the two most significant discriminators of the outcome. Therefore, to avoid overdiagnosis and overtreatment of ccRCC patients due to the low accuracy of regular histological test of RMB samples, a high FG biomarker test is required. The development and validation of the eight-gene signature made it not only applicable in regular specimen of partial or radical nephrectomy, but also predictive in ex vivo RMB samples. This could provide additional biological behavior information for both physicians and pathologist for consulting and decision making. This could also help urologists in RMB cohort to initiate an active surveillance protocol in the elderly or medically comorbid patients.

Most of the genes listed in the signature were found to be involved in differentiation and the extracellular matrix. To some extent, this result demonstrated that the signature could explain the nature of the Fuhrman grading system based upon nuclear size, and the shape and prominence of nuclei. ATOH8 is recognized as a transcriptional factor that has important roles in cell differentiation and developmental biology. ATP1A3 and CNGA1 are important in cellular energy metabolism, while CHMP4C belongs to the chromatin-modifying protein family and is associated with nucleus organization. PL2AG1S encodes an enzyme that regulates the multifunctional lysophospholipids and exists in exosomes. PPP1R1A is protein phosphatase gene, and differential expression of this gene has been reported to be associated with multistep carcinogenesis. SPOCK1 was reported to be a TGF-β target associated with epithelial–mesenchymal transition (EMT) of lung cancer and tumor proliferation and metastasis in hepatocellular and gallbladder cancer. Finally, NCRNA00116 is a long intergenic nonprotein coding RNA without a clear function annotation as yet. These genes are poorly studied in ccRCC and further research may reveal a better understanding of ccRCC and Fuhrman grading. The interactions of these genes with other well-known genes that affect biological behavior and anticancer therapy of RCC such as VHL, SMADs, BAP1, PD-L1 and CAIX remain unclear.

At last, the eight-gene classifier was proved to be predictive of prognosis. Tumor stage does not decide outcome alone. Thus in the comparison to SSIGN, we add stage into model as previous report. Results of comparing with SSIGN model showed that our model is associated with ccRCC prognosis. This reflects the reliability of our model to some extent. Since the model codes of clearcode34, ccA/ccB and S-3 score were not available to us, we cannot compare our model with them. In a previous report, 34-gene model clearcode34 did not match the ability of 90-gene model ccA/ccB. The advantage of clearcode34 is that fewer genes make it easier for applications. In the same way, our model is also easy to apply. Although our model did not match previous models, but in some special situations like insufficient sampling in biopsy for determining tumor grade, early stage tumor with serve comorbidity is suitable for active surveillance.

However, there are some limitations. First, although the discovery set was based on a global TCGA cohort, the development and RMB set were derived from the same Asian center, and thus selection bias may exist. Second, the case number of the RMB group was small and only Asians were included. Future prospective studies in large cohorts of patients of different ethnic backgrounds will be needed to fully refine the integrated grading algorithm. Third, because biopsies were taken ex vivo, the nondiagnostic rate in our study is unknown. Fourth, consider of biological heterogeneity of tumors, the limited biopsy data may not provide enough molecular information for a reliable answer. Also, six biopsies on the RMB cohort are very different from what can be obtained in a patient with a renal mass currently in practice. Moreover, ex vivo biopsies are very different from percutaneous renal biopsy in the preoperative setting. The influence of ITH should be considered validated in further studies. Therefore, our study will require validation in a cohort with preoperative biopsies in FFPE specimen.

Conclusions
After external validation, these methods may serve as examples of tools available for the diagnosis and risk-stratification of patients with ccRCC to assist in optimal individualized therapy.

Acknowledgements
The authors would like to thank the contributors to the Cancer Genome Atlas project, and Haitao Chen and Jianfeng Xu, Shenglin Huang for biostatistical assistance, Hualei Gan for central review of the pathology results. Our study is subject to the NIH Public Access Policy. This work was supported by Wu Jieping Medical Foundation grant (320.6750.1382), Shanghai Municipal Commission of Health and Family Planning grant (2014zyj0102) to Dingwei Ye; National Nature Science Foundation of China (81370073), Shanghai Rising Star Program 16QA1401100 to Yao Zhu; National Nature Science Foundation of China (81502192) to Fangning Wan.

References
1. Leibovich BC, Lohse CM, Crispen PL, et al. Histological subtype is an independent predictor of outcome for patients with renal cell carcinoma. J Urol 2010;183:1309–15.
2. Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphometric parameters in renal cell carcinoma. Am J Surg Pathol 1982;6:655–63.
3. Cheville JC, Brute ML, Zincke H, et al. Stage pT1 conventional (clear cell) renal cell carcinoma: pathological features associated with cancer specific survival. J Urol 2001;166:453–6.
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4. Zisman A, Pantuck AJ, Dorey F, et al. Improved prognostication of renal cell carcinoma using an integrated staging system. J Clin Oncol 2001;19: 1649–57.

5. Sun M, Lughezzani G, Jeldres C, et al. A proposal for reclassification of the Fuhrman grading system in patients with clear cell renal cell carcinoma. Eur Urol 2009;56:775–81.

6. Riaux-Declerq N, Karakiewicz PI, Trinh QD, et al. Prognostic ability of simplified nuclear grading of renal cell carcinoma. Cancer 2007;109: 868–74.

7. Campbell SC, Novick AC, Belldegrun A, et al. Guideline for management of the clinical T1 renal mass. J Urol 2009;182:1271–9.

8. Shannon BA, Cohen RJ, de Bruto H, et al. The value of preoperative needle core biopsy for diagnosing benign lesions among small, incidentally detected renal masses. J Urol 2008;180:1257–61.

9. Lane BR, Samplaski MK, Herts BR, et al. Renal mass biopsy—a renaissance?. J Urol 2008;179:20–7.

10. Murphy WM, Zambroni BR, Emerson LD, et al. Aspiration biopsy of the kidney. Simultaneous collection of cytologic and histologic specimens. Aspiration biopsy of the kidney. Simultaneous collection of cytologic and histologic specimens. J Urol 1986;8:67–71.

11. Tran LG, Todd TD, Dhurandhar B, et al. Fine-needle aspiration of renal masses in adults: analysis of results and diagnostic problems in 108 cases. Diagn Cytopathol 1999;20:339–49.

12. Nadel I, Baumgartner BR, Bernardino ME. Percutaneous renal biopsies: accuracy, safety, and indications. Urol Radiol 1986;6:87–71.

13. Wunderlich H, Hindermann W, Al Mustafa AM, et al. The accuracy of 250 fine needle biopsies of renal tumors. J Urol 2005;174:44–6.

14. Brooks SA, Brannon AR, Parker JS, et al. Clear Code34: a prognostic risk predictor for localized clear cell renal cell carcinoma. Eur Urol 2014;66: 77–84.

15. Yao M, Huang Y, Shioi K, et al. A three-gene expression signature to predict clinical outcome of clear cell renal carcinoma. Int J Cancer 2008;123:1126–32.

16. Sanjmyatav J, Steiner T, Wunderlich H, et al. A specific gene expression signature characterizes metastatic potential in clear cell renal cell carcinoma. J Urol 2011;186:289–94.

17. Kosari F, Parker AS, Kube DM, et al. Clear cell renal cell carcinoma: gene expression analyses identify a potential signature for tumor aggressiveness. Clin Cancer Res 2005;11:5128–39.

18. Chen J, Zhang D, Zhang W, et al. Clear cell renal cell carcinoma associated microRNA expression signatures identified by an integrated bioinformatics analysis. J Transl Med 2013;11:169.

19. Millet I, Curros F, Serre I, et al. Can renal biopsy accurately predict histological subtype and Fuhrman grade of renal cell carcinoma?. J Urol 2012;188:1885–9.

20. Bobb DI, Zhou M, Campbell SC, et al. The impact of location and number of cores on the diagnostic accuracy of renal mass biopsy: an ex vivo study. World J Urol 2013;31:1159–64.

21. Sultan M, Amsitlaskivski V, Risch T, et al. Influence of RNA extraction: a reprise. J Urol 2008;179:20–7.

22. Reiners M, Carey VJ. Bioconductor: an open source framework for bioinformatics and computational biology. Methods Enzymol 2014;515:675–97.

23. Ishaka RR. G. R: A language for data analysis and graphics. J Comput Graph Stat 1996;5:299–314.

24. Efron H. Johnstone and Tibshirani least angle regression. Ann Stat 2004;32:407–99.

25. Frank I, Blute ML, Cheville JC, et al. An outcome prediction model for patients with clear cell renal cell carcinoma treated with radical nephrectomy based on tumor stage, size, grade and necrosis: the SSIGN score. J Urol 2002;168:2395–400.

26. Ficarra V, Novara G, Galfano A, et al. The “stage, size, grade, and necrosis” score is more accurate than the University of California Los Angeles Integrated Staging System for predicting cancer-specific survival in patients with clear cell renal cell carcinoma, BJU Int 2009;103:165–70.

27. Falck AK, Ferno M, Bendahl PO, et al. St Gallen molecular reclassification in primary breast cancer and matched lymph node metastases—aspects on distribution and prognosis for patients with luminal A tumours: results from a prospective randomised trial. BMC Cancer 2013;13:558.

28. Chen J, Dai F, Balakrishnan-Renuka A, et al. Diversification and molecular evolution of ATOH8, a gene encoding a bHLH transcription factor. PLoS One 2011;6:e23005.

29. Rawnsley DR, Xiao J, Lee JS, et al. The transcription factor Atonal homolog 8 regulates Gata4 and Friend of Gata-2 during vertebrate development. J Biol Chem 2013;288:24242–7.

30. Inoue C, Bae SK, Takatsuka K, et al. Math6, a bHLH gene expressed in the developing nervous system, regulates neuronal versus glial differentiation. Genes Cells 2001;6:977–86.

31. Crambert G, Hasler U, Beghag AT, et al. Transport and pharmacological properties of nine different human Na, K-ATPase isozymes. J Biol Chem 2000;275:1976–86.

32. Dryja TP, Finn JT, Peng YW, et al. Mutations in the gene encoding the alpha subunit of the rod cGMP-gated channel in autosomal recessive retinitis pigmentosa. Proc Natl Acad Sci U S A 1995;92:10177–81.

33. Morita E, Gelf LA, Karren MA, et al. Human ESCRT-III and VP45 proteins are required for centrosome and spindle maintenance. Proc Natl Acad Sci U S A 2010;107:12889–94.

34. Prunotto M, Farina A, Lane I, et al. Proteomic analysis of podocyte exosome-enriched fraction from normal human urine. J Proteomics 2013;82: 193–229.

35. Takakura S, Kohno T, Manda R, et al. Genetic alterations and expression of the protein phosphatase 1 genes in human cancers. Int J Oncol 2001;18:817–24.

36. Miao L, Wang Y, Xia H, et al. SPOCK1 is a novel transforming growth factor-beta target gene that regulates lung cancer cell epithelial-mesenchymal transition. Biochem Biophys Res Commun 2013;440:792–7.

37. Li Y, Chen L, Chan TH, et al. SPOCK1 is regulated by CHD1L and blocks apoptosis and promotes HCC cell invasiveness and metastasis in mice. Gastroenterology 2013;144:779–91.e4.

38. Shu YJ, Weng H, Ye YY, et al. SPOCK1 as a potential cancer prognostic marker promotes the proliferation and metastasis of gallbladder cancer cells by activating the PI3K/AKT pathway. Mol Cancer 2015;14:12.

39. Strauberg RL, Feingold EA, Grouse LH, et al. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci U S A 2002;99:16899–903.

40. Gossage I, Eisen T, Maher ER. VHL, the story of a tumour suppressor gene. Nat Rev Cancer 2015;15:55–64.

41. Jingushi K, Ueda Y, Kita K, et al. miR-629 targets TRIM33 to promote TGFbeta/Smad signaling and metastatic phenotypes in ccRCC. Mol Cancer Res 2015;13:565–74.

42. Wang SS, Gu YF, Wolff N, et al. Bap1 is essential for kidney function and cooperates with Vhi in renal tumorigenesis. Proc Natl Acad Sci U S A 2014;111:16538–43.

43. Massari F, Santoni M, Ciccarese C, et al. PD-1 blockade therapy in renal cell carcinoma: current studies and future promises. Cancer Treatment Rev 2015;41:114–21.

44. Lamers CH, Steijger S, van Steenbergen S, et al. Treatment of metastatic renal cell carcinoma with CAIX CAR-engineered T cells: clinical evaluation and management of on-target toxicity. Mol Ther 2013;21:904–12.

45. Brannon AR, Reddy A, Seiler M, et al. Molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. Genes Cancer 2010;1:152–63.

46. Buttner F, Winter S, Rausch S, et al. Survival prediction of clear cell renal cell carcinoma based on gene expression similarity to the proximal tubule of the nephron. Eur Urol 2015;68:1016–20.