EBioMedicine 2 (2015) 1814–1820

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Article

A Molecular Model for Predicting Overall Survival in Patients with Metastatic Clear Cell Renal Carcinoma: Results from CALGB 90206 (Alliance)

Hyung L. Kim [a,*,1], Susan Halabi [b,1], Ping Li [a], Greg Mayhew [c], Jeff Simko [d], Andrew B. Nixon [c], Eric J. Small [d], Brian Rini [e], Michael J. Morris [f], Mary-Ellen Taplin [g], Daniel George [b], for the Alliance for Clinical Trials in Oncology

[a] Cedars-Sinai Medical Center, Los Angeles, CA, United States
[b] Department of Biostatistics and Bioinformatics, and Alliance Statistics and Data Center, Duke University, Durham, NC, United States
[c] GeneCentric Diagnostics, Durham, NC, United States
[d] University of California at San Francisco, San Francisco, CA, United States
[e] Cleveland Clinic Taussig Cancer Institute, Cleveland, OH, United States
[f] Memorial Sloan Kettering Cancer Center, New York, NY, United States
[g] Dana-Farber Cancer Institute, Boston, MA, United States

ARTICLE INFO

Article history:
Received 5 August 2015
Received in revised form 6 September 2015
Accepted 7 September 2015
Available online 8 September 2015

Keywords:
Renal cell carcinoma
Prognostic markers
Prognostic signature
Expression profile

ABSTRACT

Background: Prognosis associated with metastatic renal cell carcinoma (mRCC) can vary widely.
Methods: This study used pretreatment nephrectomy specimens from a randomized phase III trial. Expression levels of candidate genes were determined from archival tumors using the OpenArray® platform for TaqMan® RT-qPCR. The dataset was randomly divided at 2:1 ratio into training (n = 221) and testing (n = 103) sets to develop a multigene prognostic signature.
Findings: Gene expressions were measured in 324 patients. In the training set, multiple models testing 424 candidate genes identified a prognostic signature containing 8 genes plus MSKCC clinical risk factors. In the testing set, the time dependent (td) AUC for a prognostic model containing the 8 genes with and without MSKCC risk factors were 0.72 and 0.69, respectively. The tdAUC for the clinical risk factors alone was 0.61. Additional primary mRCCs from patients with mRCC (n = 12) were sampled in multiple sites and standard deviations of gene expressions within a tumor were used as a measure of heterogeneity. All 8 genes in the final prognostic model met our criteria for minimal heterogeneity.
Conclusions: A molecular prognostic signature based on 8 genes was developed and is ready for external validation in this patient population and other related settings such as nonmetastatic RCC.

1. Introduction

Despite being uniformly fatal, survival associated with metastatic renal cell carcinoma (RCC) can vary widely, from a few months to several years. While clinical findings and histomorphologic characteristics of the tumor can provide reasonable estimates of survival, greater precision is needed. Whether molecular signatures from the primary tumor can provide that increased precision is not known, but if so, this approach might prove useful for patient counseling, treatment planning, and determining clinical trial eligibility.

Many prior studies have reported biomarkers and molecular signatures for predicting survival (Takahashi et al., 2001; Sultmann et al., 2005; Kosari et al., 2005; Jones et al., 2005; Zhao et al., 2006; Cancer Genome Atlas Research N, 2013; Brannon et al., 2010). Unfortunately, the majority of these studies included patients with both localized and metastatic RCC. Most localized RCCs have a favorable prognosis, and the American Urologic Association recommends observation as a valid management for many small renal cancers (Campbell et al., 2009). In contrast, metastatic RCC is nearly always fatal. Therefore, it is not surprising that molecular signatures can be readily identified for separating these patients into two prognostic groups. In contrast, there is a paucity of studies reporting prognostic molecular signatures that can be applied solely to metastatic RCC.

Prior studies of biomarkers from cytoreductive nephrectomies are also limited by small sample sizes and have usually focused on a limited

* Corresponding author at: Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048, United States.
E-mail address: kimhl@cshs.org (H.L. Kim).
[1] Co-first authors; these authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ebiom.2015.09.012

numbers of candidate markers assessed by immunohistochemistry (Vasselli et al., 2003; Miyake et al., 2009; Kusuda et al., 2013; Kim et al., 2005). To develop prognostic biomarkers for metastatic RCC, there remains a need for discovery studies using multi-institutional tissue banks from well-characterized patients whose treatment and outcomes were rigorously annotated. Therefore, we report a gene expression-based prognostic signature developed using primary untreated RCC collected as part of Cancer and Leukemia Group B (CALGB) 90206, a randomized phase III trial of interferon alpha (INF) vs. INF plus bevacizumab in patients with metastatic or unresectable RCC (Rini et al., 2010). CALGB is now a part of the Alliance for Clinical Trials in Oncology. CALGB 90206 demonstrated statistically longer progression-free survival (PFS) but no statistical overall survival (OS) benefit for patients treated with the combination therapy. CALGB 90206 was used to develop a prognostic signature for predicting OS.

2. Materials and Methods

2.1. Patient Population

Patients eligible for CALGB 90206 had metastatic or unresectable RCC with a clear cell histology, Karnofsky performance status ≥ 70%, and adequate organ function. Prior chemotherapy for metastatic disease was not permitted. A stratified random block design was used with the stratification factors of nephrectomy and number of adverse risk factors as defined by the Motzer criteria (Motzer et al., 2002). All details of the clinical trial are published elsewhere (Rini et al., 2008, 2010). Each participant signed an IRB-approved, protocol-specific informed consent in accordance with federal and institutional guidelines. Data collection and statistical analyses were conducted by the Alliance Statistics and Data Center.

2.2. RNA Extraction

Using tumors received as part of CALGB 90206, H&E stains were made of samples received by CALGB and were reviewed by a genitourinary pathologist (JS) who annotated the outline of the tumor on a digital image, which was used to macrodissect the tumor for RNA extraction. All assay work was performed at Cedars Sinai (HK). Our method for RNA extraction from FFPE renal tumors has been previously described (Glenn et al., 2010). Briefly, RNA was extracted from six 10-μm sections when archival blocks were available. Some participating sites chose to send unstained slides, and three 5-μm sections were used.

Tumor sections were placed in 2.0-ml RNase-free Eppendorf tubes. Sections were treated twice with 1.9 ml xylene for 5 min (min) at 55 °C while rocking. The sections were washed twice with 100% ethanol. RNA was extracted from the paraffin samples using the MasterPure™ RNA Purification Kit (Epicenter Biotechnologies, Madison, WI, USA). In an attempt to further increase RNA yield, FFPE samples were treated with 200 μg Proteinase K for 3 h at 55 °C. RNA was then treated with 20 units DNase I (Ambion, Austin, TX, USA) for 30 min and checked for residual genomic DNA by TaqMan RT-PCR targeting ACTB. If there was measurable DNA after 34 PCR cycles using 50 ng input RNA, the samples were treated with 20 units DNase I for an additional 15 min, and the assay for residual DNA was repeated. The final RNA concentration (A260:0.025) and purity (A260:A280 ratio) was measured using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3. Reverse Transcription

Reverse transcription (RT) was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY) following the manufacturer's recommendation. Each 10 μl RT reaction contained 150 ng of total RNA (75 ng or 37.5 ng was used for cases with lower RNA yield), 1 μl of 10X RT buffer, 0.5 μl of 25X dNTP mixture, 1 μl of 10X random reverse primers, 1 μl of 10X gene-specific reverse primers (1 μM) and 0.5 μl of MultiScribe RT (50 U/μl). The 10 μl reactions were incubated in a Life Technologies Thermocycler for 10 min at 25 °C, 2 h at 37 °C, 5 min at 85 °C and then held at 4 °C. 10X pooled gene-specific reverse primers (1 μM) were prepared by combining equal volumes of each 500 μM reverse primer (primers for all candidate genes were pooled). The same primers were used for gene-specific RT, preamplification and qPCR. The candidate genes were identified from a literature search for prognostic and predictive gene expressions determined from microarrays and tissue microarrays. Key genes involved in pathways known to be important in RCC were also included.

The preamplification was performed using TaqMan® PreAmp Master Mix Kit (Life Technologies, Grand Island, NY) as previously described (Li et al., 2013). Each 5 μl of preamplification reaction included 2.5 μl of 2X TaqMan® PreAmp Master Mix, 1.25 μl of 0.09X pooled TaqMan assay mix and 1.25 μl of cDNA. The reactions were incubated in an Applied Biosystems Thermocycler for 10 min at 95 °C followed by 13–15 cycles (depending on starting RNA) of 95 °C for 15 s and 60 °C for 4 min and then held at 4 °C. Pooled TaqMan assays (0.09X) were prepared by combining equal volumes of each 20X TaqMan assay (needed for PCR on the Openarray®, 218 assays for each set). Each cDNA was preamplified on two sets of 218 pooled assays. Preamplified cDNA products were diluted 10 times with 1X TE buffer for storage and PCR.

2.4. Real-time PCR on OpenArray Platform

Two sets of TaqMan® OpenArray® Real-Time PCR Plates (Life Technologies, Grand Island, NY) were made using custom-designed primers and probes. Our strategy for designing TaqMan® assays has been described (Li et al., 2013). Briefly, gene sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Repeats and low complexity sequences, and SNPs were masked. The resulting sequences were sent to Life Technologies for custom design of primers and probes using their proprietary software. Each PCR target was blasted to avoid amplification of unintended targets. When multiple isoforms existed, targets were selected in regions common to all isoforms. All primers were designed to generate amplicons less than 100 base pairs (Supplemental Table 1).

Each OpenArray® Real-Time PCR Plate contains 218 TaqMan® assays. Diluted preamplified cDNA (10 μl) was mixed with 10 μl of TaqMan® OpenArray® Real-Time PCR Master Mix (Life Technologies, Grand Island, NY). The mixed cDNA samples were dispensed into an OpenArray® 384-well Sample Plate (Life Technologies, Grand Island, NY) with each sample placed into 4 wells at 5 μl per well. cDNA samples were then dispensed into OpenArray® Real-Time PCR Plates using OpenArray® AccuFill™ System (Life Technologies, Grand Island, NY). The real-time PCR reactions were incubated in an OpenArray® NT Cycler system for 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min and then held at 4 °C.

Post-acquisition data processing generated fluorescence amplification for each assay, from which cycle threshold (CT) were computed and used for further data analysis. Each gene was normalized using 6 previously identified reference genes, which were measured in quadruplicate (Glenn et al., 2007). Each PCR plate had a control cDNA and ACTB amplification was always with 0.5 CT of the expected value. Tumor expression data were generated for 430 candidate genes identified from a literature search. C_T levels were normalized with six housekeeping genes. Expression levels that were too low to detect were imputed to 30, which corresponded to a single copy of a gene in the assay chamber (Li et al., 2013).

2.5. Statistical Analysis

The primary end point used for this analysis was overall survival (OS), defined as the time from randomization to date of death of any cause. The date of data cutoff for the clinical trial was March 24, 2009 and median followup among surviving patients was 46.2 months. The dataset was

randomly divided at 2:1 ratio into training (n = 221) and testing (n = 103) sets to develop a multigene prognostic signature. Training and testing samples were normalized together prior to random allocation. To adjust for any lingering batch effects, we calculated gene means and standard deviations within each batch, then centered and scaled samples to have within-batch gene means 0 and standard deviations 1. The comparative C_T method was used to analyze the data (also referred to as $\Delta\Delta C_T$) (Schmittgen and Livak, 2008).

There were 12 individuals with two samples from different regions of the tumor that were used to assess heterogeneity. We used the median of the 12 standard deviations as a measure of stability and chose a cut-point of 0.78. K-means clustering algorithm was utilized to identify the threshold for the stable genes.

2.6. Model Building

Several steps were used for model building to help prioritize genes for the multivariable model of OS. First, univariate proportional hazards models were fit in the training set to test for the prognostic importance of the 424 genes in predicting overall survival. Twenty-one genes had q-value (false discovery rate) <0.05 in the univariate scans and were selected for the multivariable model. In the second step, the least absolute shrinkage and selection operator (LASSO) penalty was used to identify important genes for the multivariable model (Tibshirani, 1997). The main advantage of using penalized methods is that they produce sparse regression coefficients, and the selection of important prognostic factors does not depend on statistical significance. The regularization parameter was chosen to minimize the Schwarz Information Criterion. The 95% CI for the LASSO was derived by adopting the perturbation method proposed by Minnier and extending their work to the Cox's regression (Minnier et al., 2011; Lin and Halabi, in press). In the final step, all possible multivariable models of eight genes from 21 potentially important genes were fit to the training data (203,490 multivariable models). The top 100 models were ranked by the concordance index and the highest time-dependent AUC (tdAUC) and the final model was chosen accordingly. A risk score was calculated for each patient using the estimated regression coefficients from the training set.

2.7. Validation

The parameter estimates from the locked final model were applied to the testing set and a risk score was computed for every patient. The performance of the final model was assessed by computing the tdAUC. In addition, the tdAUC was computed for the eight gene model and for the model containing only the Memorial Sloan Kettering Cancer Center (MSKCC) clinical risk factors. The 95% CI for the tdAUC was computed using the bootstrapped approach. The final model was validated with the risk score as a continuous variable. Tertiles based on the training set were identified and applied to the risk score in the testing set. Patients were grouped into low, intermediate or high-risk groups. The final model was validated by one of the authors (SH) who did not have access to the training set. The Kaplan-Meier product-limit method was used to estimate the overall survival distribution by the different risk groups and the log-rank statistic was used to test if the three-risk groups have different survival outcomes. All statistical analyses for model development and validation were performed using the R package.

2.8. Funding Support

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under Award Numbers U10CA180821 and U10CA180882 (to the Alliance for Clinical Trials in Oncology), CA31946, CA33601, R01CA133072, U01CA157703 and R01CA155296. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funders had no role in study design, data collection, data analysis, interpretation, and writing of the report.

3. Results

CALGB 90206 enrolled 732 patients in the United States and Canada between October 2003 and July 2005. The primary outcomes from the treatment trial have been previously reported (Rini et al., 2008, 2010). Fig. 1 presents the REMARK diagram. Consents for use of tissue for correlative studies were obtained from 92% (676/732) of patients. The eligibility for the parent trial required primary tumor tissue to be available. However, tissue submission was not required for patients to start treatment. Paraffin-embedded tumor blocks or unstained slides were received for 395 patients. All tissues were H&E stained and centrally reviewed by a single GU pathologist (J.S.) The final analysis was based on 324 patients with available specimens.

Baseline patient characteristics are summarized in Table 1 for the training and testing sets. The baseline clinical characteristics for patients in the training and testing sets were comparable and similar to that of the entire cohort enrolled on CALGB 90206 with a few exceptions. Given that most tumor tissue came from cytoreductive nephrectomies, the percent of patients having nephrectomy was higher in study subjects (99% vs 73%).

Using the training set (n = 221), all 424 genes were evaluated in the proportional hazards models in predicting OS. Univariate analysis for the 424 genes using the entire cohort of 324 subjects is presented in Supplemental Table 2. The top 21 prognostic genes are presented in Table 2. These genes with q-value <0.05 were considered as candidate genes in the multivariable analysis. The hazard ratios (HR) for normalized $\Delta\Delta C_T$

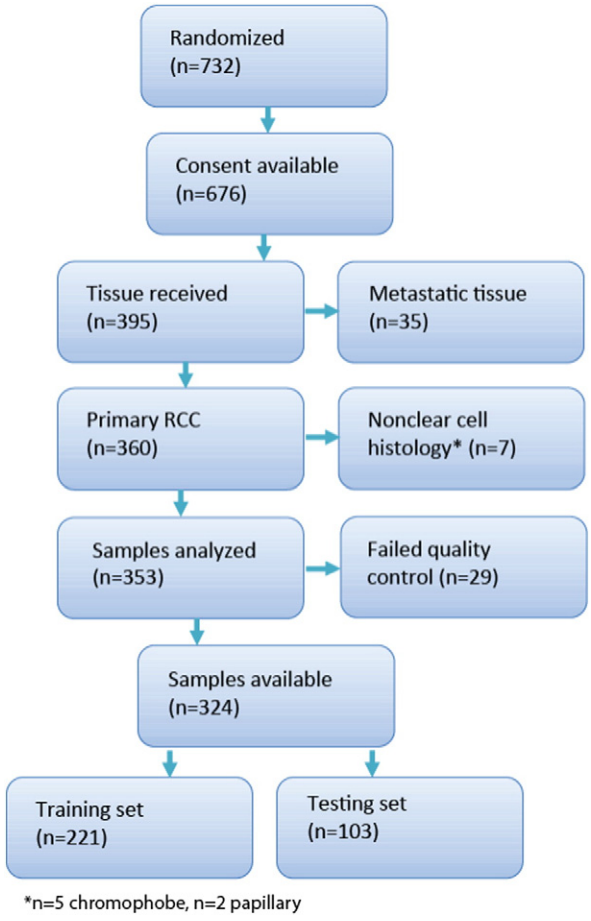


Fig. 1. REMARK diagram. The diagram accounts for each patient in the parent clinical trial and the availability of their tumor tissue for this study.

Table 1
Patient characteristics.

	Tissues not available (n = 379)	Training Set (n = 221)	Testing Set (n = 103)	Total (n = 732)
Median age, years	62	61	63	62
(25th, 75th percentile)	(56–70)	(55–69)	(56–71)	(55–70)
Gender (%)				
Male	267 (70)	152 (69)	69 (67)	505 (69)
Female	112 (30)	69 (31)	34 (33)	227 (31)
Nephrectomy (%)	276 (73)	218 (99)	102 (99)	620 (85)
ECOG performance status (%)				
0	122 (33)	92 (42)	36 (35)	259 (36)
1	225 (60)	112 (51)	58 (56)	414 (57)
2	27 (7)	14 (6)	8 (8)	50 (7)
Unknown	5 (0)	3 (1)	1 (1)	9
Common Sites of Metastases* (%)				
Lung	248 (66)	165 (75)	75 (73)	507 (69)
Lymph node	130 (34)	89 (40)	29 (28)	259 (35)
Bone	32 (33)	59 (27)	22 (21)	213 (29)
Liver	95 (25)	32 (14)	15 (15)	147 (20)
Number of Risk Factors** (%)				
0 (favorable)	101 (27)	61 (28)	21 (20)	192 (26)
1–2 (intermediate)	231 (61)	144 (65)	74 (73)	465 (64)
> = 3 (poor)	47 (12)	16 (7)	8 (8)	75 (10)

* Not mutually exclusive.
** MSKCC adverse clinical risk factors.

values are provided. Lower $\Delta\Delta C_T$'s corresponds to higher expression levels, therefore HRs <1 indicate higher expression level and decreased risk of death.

3.1. Multivariable Model

Using LASSO 8 genes were identified. Therefore, 8 genes were determined as the optimal size for the final model and all possible models of eight genes out of 21 significant genes were fit. For illustrative purposes, the Kaplan-Meier plots are provided for each of the eight genes that were included in the final model. The genes are dichotomized by the observed medians into high and low expression groups (Supplemental Fig. 1). In the parent clinical trial, the number of MSKCC clinical risk factors was used as a stratification factor in the randomization. Therefore, MSKCC clinical risk factors were included in the final multivariable model (Table 3). In the training set, the tdAUC for the final 8-gene model with the MSKCC risk factors was 0.71 (95% CI = 0.59–0.73). For *CRYL1*, *PCNA and CDK1*, decreased expression levels were associated with worse OS; however, for *TRAF2*, *USP6NL*, *CEP55*, *HGF and HSD17B10*, the inverse association was observed. The final model was assessed for calibration (internal validation). The predicted probabilities at 18 (median OS in the clinical trial)-, and 24-months from the model were close to the observed probability of survival (Supplement Fig. 2).

Table 2
Top 21 prognostic genes in the training set*.

Genes	HR**	CI**	p-value	q-value
MCM2	0.7	(0.60–0.82)	<0.0001	0.00297
CCNB1	0.74	(0.64–0.86)	<0.0001	0.01036
TOP2A	0.75	(0.64–0.87)	0.00015	0.01036
NPM3	0.74	(0.63–0.86)	0.00016	0.01036
CEP55	0.75	(0.64–0.87)	0.00025	0.01282
FSCN1	0.76	(0.65–0.88)	0.00036	0.0152
KIAA0101	0.76	(0.65–0.89)	0.00052	0.01912
CRYL1	1.3	(1.11–1.53)	0.00088	0.02507
CDK1	0.77	(0.65–0.90)	0.00098	0.02507
KIF23	0.78	(0.67–0.90)	0.00105	0.02507
L1CAM	0.78	(0.68–0.91)	0.00108	0.02507
TRAF2	0.78	(0.67–0.91)	0.00127	0.02582
ANLN	0.77	(0.65–0.90)	0.00131	0.02582
KLK1	0.77	(0.66–0.91)	0.0017	0.02968
HGF	0.78	(0.66–0.91)	0.00174	0.02968
USP6NL	0.79	(0.68–0.92)	0.00265	0.04073
PCNA	0.8	(0.69–0.93)	0.00286	0.04073
MELK	0.77	(0.65–0.92)	0.0029	0.04073
PRC1	0.78	(0.66–0.92)	0.00312	0.04073
POLR2B	0.8	(0.69–0.93)	0.00322	0.04073
HSD17B10	0.8	(0.69–0.93)	0.00334	0.04073

* Genes in our final prognostic model are in bold.
** HR, hazard ratio; CI, 95% confidence interval.

3.2. Testing Set

Using the final model, risk score was highly predictive of OS with a tdAUC = 0.72 (95% CI = 0.66–0.78). The testing set was divided into equal thirds to generate cutoffs for low, intermediate, and high risk groups. Median OSs were 38 (95% CI = 26-not reached), 21 (95% CI = 14–32) and 13 (95% CI = 9–19) months, respectively ($p < 0.001$, Fig. 2a). For comparison, the 8-gene model without the MSKCC clinical risk factors was similarly applied to the testing set (Fig. 2b) and had a tdAUC of 0.69 (95% CI = 0.62–0.72). Fig. 2c presents the Kaplan-Meier survival curves for risk groups based on number of MSKCC clinical risk factors. It is noteworthy that the tdAUC for this model was only 0.61(95% CI = 0.54–0.69). Fig. 3 shows the AUC at 18 (Fig. 3a) and 24 months (Fig. 3b) for the three models. It is clear that the final model based on the 8 genes and the MSKCC clinical risk factors is superior to the model with MSKCC clinical risk factors alone.

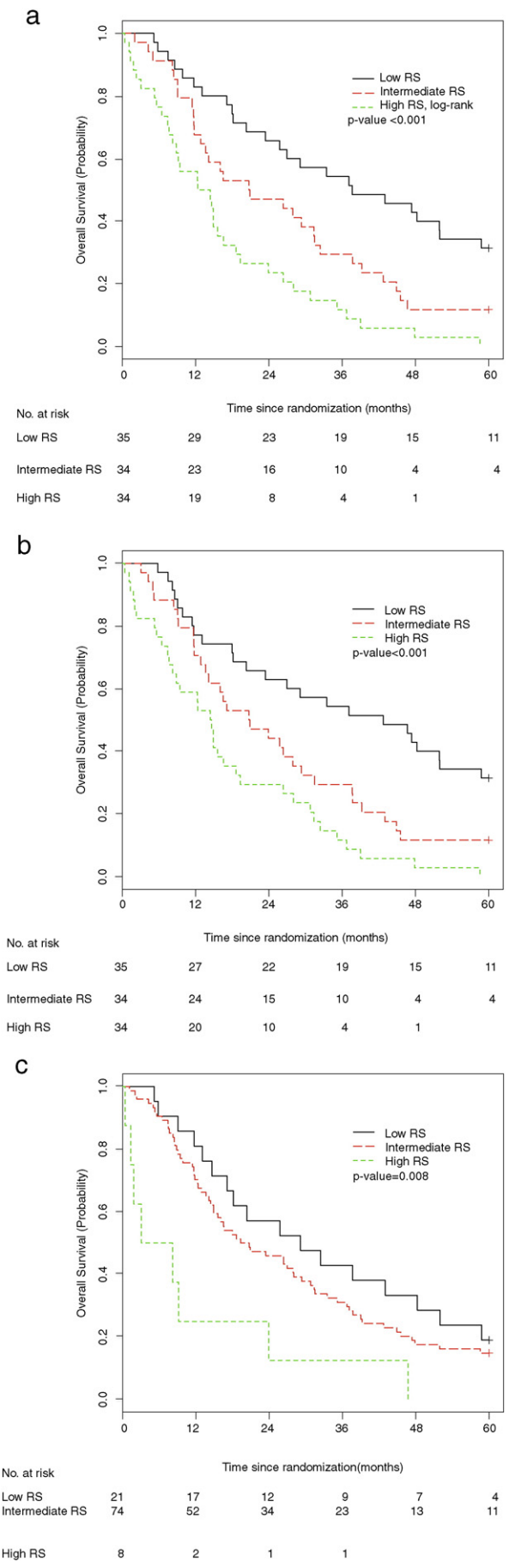
3.3. Stability Analysis

Mutations in individual renal tumors are highly heterogeneous (Gerlinger et al., 2012, 2014). Therefore, to ensure the stability of our gene signature, the expression of the 424 candidate genes were measured from two random sites using 12 primary clear cell RCCs from

Table 3
Prognostic model for predicting overall survival: training set.*

	HR*	95% CI*	p-value
CRYL1	1.428	(1.188, 1.716)	0.0001
TRAF2	0.806	(0.688, 0.945)	0.0079
USP6NL	0.914	(0.751, 1.111)	0.0101
CEP55	0.772	(0.634, 0.940)	0.0246
HGF	0.918	(0.761, 1.107)	0.1818
PCNA	1.167	(0.930, 1.464)	0.3657
CDK1	1.093	(0.870, 1.372)	0.3688
HSD17B10	0.793	(0.648, 0.971)	0.4449
1–2 RF** vs. 0	1.317	(0.939, 1.849)	0.111
> = 3 RF vs. 0	2.596	(1.467, 4.594)	0.001

* HR, hazard ratio; CI, 95% confidence interval.
** RF, MSKCC Adverse Clinical Risk Factors.



patient with metastatic disease. For each gene we calculated standard deviations for each RCC. The stability measure for each gene was the median of the 12 standard deviations. K-means clustering ($K = 2$) was used to determine a threshold (0.78) for dividing the genes into stable and unstable genes based on the stability measure (Fig. 4). Of the 424 genes, 83 genes were considered unstable. All 8 genes in our final model were confirmed to be stable. In a *post-hoc* analysis, we repeated our model building approach using stable genes only, and achieved a test AUC of 0.72, just below our locked model. This is in part due to the fact that the most significant genes in univariate analysis tended to be stable genes.

4. Discussion

Approximately one-third of patients newly diagnosed with RCC have metastatic disease, and after treatment for localized RCC, 25–50% of patients will suffer metastatic recurrence. The survival for individual patients can vary widely. Patients can be stratified into risk groups based on readily available clinical parameters such as performance status, serum lactate dehydrogenase, hemoglobin, serum calcium, and length of time between initial diagnosis and treatment (Motzer et al., 2002). The number of MSKCC adverse clinical risk factors was used to stratify the randomization for the parent clinical trial of this study, CALGB 90206. Unfortunately, MSKCC adverse clinical risk factors only had modest prognostic ability in our population with a tdAUC of 0.61, demonstrating the need for developing predictive markers with higher precision.

This study developed a multimarker prognostic signature from a large multicenter phase III, randomized clinical trial in RCC in which eligibility was clearly defined and outcomes rigorously recorded. In addition to recruiting a diverse range of patients, a multicenter study is less susceptible to systematic bias introduced by institution-specific procedures for tissue handling and storage. Importantly, the prognostic signature was obtained using formalin-fixed, paraffin-embedded tumors, which are routinely collected and stored in all pathology departments. For clear cell RCC, tumor tissue is routinely available from cytoreductive nephrectomy or diagnostic biopsy. To the best of our knowledge, there are no prior reports of a multimarker molecular signature developed from a multicenter, phase III clinical trial of RCC.

CALGB 90206 randomized patients with newly diagnosed clear cell RCC to IFN or IFN plus bevacizumab (Rini et al., 2008). The primary endpoint was overall survival, and secondary endpoints were progression free survival and safety. The majority (85%) of patients underwent a cytoreductive nephrectomy, and 90% had favorable or intermediate prognosis based on number of MSKCC adverse clinical risk factors. At the interim analysis, the median PFS was 5.2 months in the IFN group and 8.5 months in the IFN plus bevacizumab group ($p < 0.0001$). However, no statistically significant difference in OS was observed between the two groups. The median OS was 17.4 months in the IFN group and 18.3 months in the combination arm (Rini et al., 2010). Furthermore, subset analysis failed to identify any clinical variable associated with treatment response. Therefore, no clinical variable other than MSKCC adverse clinical risk factors were included in our final model.

An important limitation of prior biomarkers studies is that they included all stages of RCC (Takahashi et al., 2001; Sultmann et al., 2005; Kosari et al., 2005; Jones et al., 2005; Zhao et al., 2006; Cancer Genome Atlas Research N, 2013; Brannon et al., 2010), limiting their applicability to patients with metastatic disease. However, many of these studies used an unbiased, genome-wide approach to discovery of prognostic markers, and provided a wealth of candidate gene expression markers

Fig. 2. Kaplan–Meier survival curves for prognostics models. (a) Final model containing 8-genes plus MSKCC clinical risk factors. (b) 8-gene-only prognostic model. (a & b) Multivariable model developed using the training set was used to assign risk scores to the testing set. Cutoffs for risk groups were defined by dividing the training set into tertiles. (c) Risk groups defined by number of MSKCC clinical risk factors.

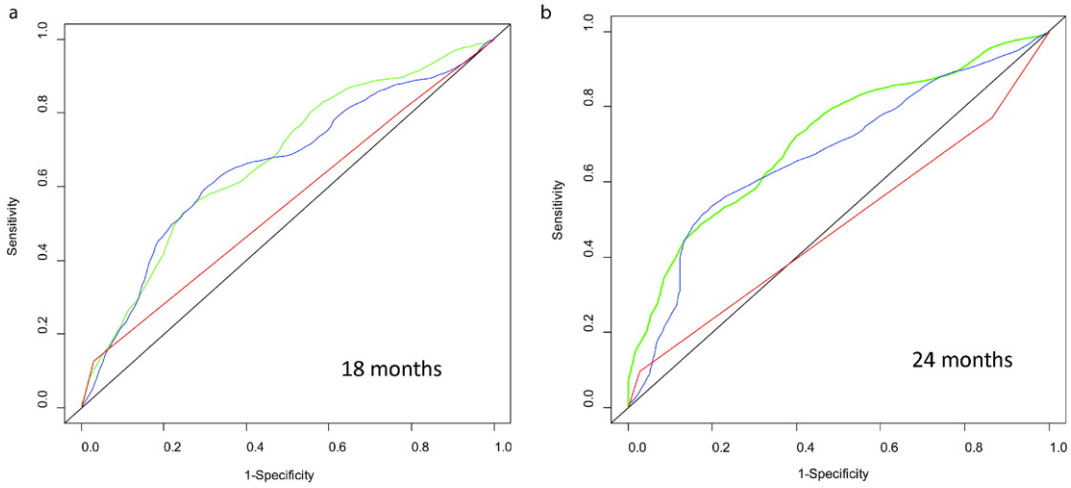


Fig. 3. AUC plots at 18-months (a) and 24 months (b). The green line represents the final model, blue line is the model with 8 genes, and the red line is the MKSCC clinical risk factors only.

for us to evaluate. An increased understanding of pathways and mechanisms driving clear cell RCC provided additional candidate markers. In univariate analysis, we found that 21 of the candidate biomarkers were significant predictors of OS using $q < 0.05$ (Table 2 and Supplemental Table 2). These results are validation of prior discovery studies of prognostic markers.

Our multivariable analysis identified an 8-gene model of OS. Following VHL inactivation, *PBRM1* is the second major gene in ccRCC, with truncating mutations in 41% of cases (Varela et al., 2011). Genes in pathways deregulated following *PBRM1* knockdown in RCC cell lines were included as candidate genes in this study. In our final prognostic model, 4 genes (*CRYL1*, *HSD17B10*, *CEP55* and *HGF*) were *PBRM1* related genes; 3 (*CRYL1*, *HSD17B10 and CEP55*) were also differentially expressed when comparing ccRCC to normal kidney (Tun et al., 2010). HGF, which binds the proto-oncogene c-MET, has been linked to invasiveness and VHL inactivation in ccRCC (Horie et al., 1999; Koochekpour et al., 1999; Harshman and Choueiri, 2013). Both *TRAF2* (Vasselli et al., 2003) and *USP6NL* (Zhao et al., 2006) were previously identified as prognostic genes is microarray-based studies of RCC. *PCNA* was included as a candidate gene because it is a classic marker of proliferation and has been previously associated with RCC prognosis (Nogueira and Kim, 2008). *CDK1*, a cell cycle regulator, was included as a candidate gene because it was previously reported to predict response to antiangiogenic and epidermal growth factor targeted therapy in RCC (Tsavachidou-Fenner et al., 2010). When generating our prognostic signature, genes were favored that provided independent and non-redundant prognostic information. Therefore, it is not surprising that our 8 genes have been associated with a wide range of functions important to cancer progression such as proliferation (*CEP55*, *PCNA*, *CDK1*), apoptosis (*TRAF2*), metabolism (*CRYL1*, *HSD17B10*) and invasion (*HGF*) (http://www.ncbi.nlm.nih.gov/gene, 2015).

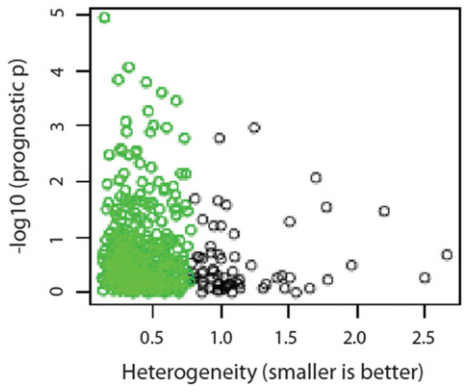


Fig. 4. Evaluation of tumor heterogeneity. The tumor was sampled in two separate areas and gene expressions were determined by qPCR. Heterogeneity was defined as the median of standard deviations determined from sampling each tumor twice. A threshold of 0.78 for unacceptable heterogeneity (black circles) was determined using the K-means clustering algorithm with $k = 2$.

The genetic heterogeneity of RCC is well documented (Gerlinger et al., 2012, 2014). However, the clonal evolutionary tree has a common "trunk" that links all genomic mutations. In addition, there are common histologic features that pathologists use to classify renal tissue as RCC. Therefore, it is reasonable to expect that there are markers, particularly expression markers that directly reflect the phenotype of RCC. To generate a signature that was less sensitive to sampling artifacts produced by tumor heterogeneity, we performed a separate analysis using untreated primary tumors from metastatic clear cell RCC patients that were sampled in two different areas. Genes with heterogeneous expression within individual patients were excluded from consideration in our multimarker models.

There are several strengths of the present analysis. First, the trial had a large number of tissue specimen available. Second, the data were from a randomized multi-institutional phase III trial. Results of multicenter studies are convincing because tissues are less susceptible to systemic bias resulting from institution-specific tissue-handling protocols and are more likely to be representative. The parent trial clearly defines the patient cohort for which the signature can be applied. Furthermore, patient treatment and follow-up have been rigorously recorded, with oversight from a highly developed coordinating center.

An important limitation of the study is the absence of an external validation cohort. What remains unresolved is whether tissue handling introduced systematic bias or that the assay is reproducible by other investigators. However, the tissue was received, stored and released by the CALGB pathology central office, which has extensive experience with biobanking for clinical trials. The qPCR assay is well established and robust, and routinely used in commercial laboratories. Future studies should further validate our prognostic signature and establish its clinical utility to impact patient outcome by improving survival or deceasing treatment-related morbidity. Additional studies can qualify the signature for use with additional, related cohorts of RCC such as patients treated for clinically localized RCC who remain at high risk for recurrence.

5. Conclusion

A molecular prognostic signature based on 8 genes and MSKCC adverse clinical risk factors was developed and tested using tissue from a phase III trial and predicts OS in patients with metastatic clear cell RCC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.09.012.

References

Brannon, A.R., Reddy, A., Seil er, M., Arreola, A., Moore, D.T., Pruthi, R.S., et al., 2010. Molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. Genes Cancer 1 (2), 152–163.

Campbell, S.C., Novick, A.C., Belldegrun, A., Blute, M.L., Chow, G.K., Derweesh, I.H., et al., 2009. Guideline for management of the clinical T1 renal mass. J. Urol. 182 (4), 1271–1279.

Cancer Genome Atlas Research N, 2013. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 499 (7456), 43–49.

Gene [Internet]. National Center for Biotechnology Information [cited April 3, 2015] Available from: http://www.ncbi.nlm.nih.gov/gene.

Gerlinger, M., Rowan, A.J., Horswell, S., Larkin, J., Endesfelder, D., Gronroos, E., et al., 2012. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N. Engl. J. Med. 366 (10), 883–892.

Gerlinger, M., Catto, J.W., Orntoft, T.F., Real, F.X., Zwarthoff, E.C., Swanton, C., 2014. Intratumour heterogeneity in urologic cancers: from molecular evidence to clinical implications. Eur. Urol.

Glenn, S.T., Jones, C.A., Liang, P., Kaushik, D., Gross, K.W., Kim, H.L., 2007. Expression profiling of archival renal tumors by quantitative PCR to validate prognostic markers. Biotechniques 43 (5) (639–40, 42–3, 47).

Glenn, S.T., Head, K.L., Teh, B.T., Gross, K.W., Kim, H.L., 2010. Maximizing RNA yield from archival renal tumors and optimizing gene expression analysis. J. Biomol. Screen. 15 (1), 80–85.

Harshman, L.C., Choueiri, T.K., 2013. Targeting the hepatocyte growth factor/c-Met signaling pathway in renal cell carcinoma. Cancer J. 19 (4), 316–323.

Horie, S., Aruga, S., Kawamata, H., Okui, N., Kakizoe, T., Kitamura, T., 1999. Biological role of HGF/MET pathway in renal cell carcinoma. J. Urol. 161 (3), 990–997.

Jones, J., Otu, H., Spentzos, D., Kolia, S., Inan, M., Beecken, W.D., et al., 2005. Gene signatures of progression and metastasis in renal cell cancer. Clin. Cancer Res. 11 (16), 5730–5739.

Kim, H.L., Seligson, D., Liu, X., Janzen, N., Bui, M.H., Yu, H., et al., 2005. Using tumor markers to predict the survival of patients with metastatic renal cell carcinoma. J. Urol. 173 (5), 1496–1501.

Koochekpour, S., Jeffers, M., Wang, P.H., Gong, C., Taylor, G.A., Roessler, L.M., et al., 1999. The von Hippel–Lindau tumor suppressor gene inhibits hepatocyte growth factor/scatter factor-induced invasion and branching morphogenesis in renal carcinoma cells. Mol. Cell. Biol. 19 (9), 5902–5912.

Kosari, F., Parker, A.S., Kube, D.M., Lohse, C.M., Leibovich, B.C., Blute, M.L., et al., 2005. Clear cell renal cell carcinoma: gene expression analyses identify a potential signature for tumor aggressiveness. Clin. Cancer Res. 11 (14), 5128–5139.

Kusuda, Y., Miyake, H., Behnsawy, H.M., Fukuhara, T., Inoue, T.A., Fujisawa, M., 2013. Prognostic prediction in patients with metastatic renal cell carcinoma treated with sorafenib based on expression levels of potential molecular markers in radical nephrectomy specimens. Urol. Oncol. 31 (1), 42–50.

Li, P., Grigorenko, E., Funari, V., Enright, E., Zhang, H., Kim, H.L., 2013. Evaluation of a high-throughput, microfluidics platform for performing TaqMan qPCR using formalin-fixed paraffin-embedded tumors. Bioanalysis 5 (13), 1623–1633.

Lin, C.Y., Halabi, S., 2015. A simple method for deriving the confidence regions for the penalized Cox's model via the minimand perturbation. Communications in Statistics: Theorey and Methods (in press).

Minnier, J., Tian, L., Cai, T., 2011. A Perturbation Method for Inference on Regularized Regression Estimates. J. Am. Stat. Assoc. 106 (496), 1371–1382.

Miyake, H., Sakai, I., Muramaki, M., Kurahashi, T., Takenaka, A., Fujisawa, M., 2009. Prediction of response to combined immunotherapy with interferon-alpha and low-dose interleukin-2 in metastatic renal cell carcinoma: expression patterns of potential molecular markers in radical nephrectomy specimens. Int. J. Urol. 16 (5), 465–471.

Motzer, R.J., Bacik, J., Murphy, B.A., Russo, P., Mazumdar, M., 2002. Interferon-alfa as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma. J. Clin. Oncol. 20 (1), 289–296.

Nogueira, M., Kim, H.L., 2008. Molecular markers for predicting prognosis of renal cell carcinoma. Urol. Oncol. 26 (2), 113–124.

Rini, B.I., Halabi, S., Rosenberg, J.E., Stadler, W.M., Vaena, D.A., Ou, S.S., et al., 2008. Bevacizumab plus interferon alfa compared with interferon alfa monotherapy in patients with metastatic renal cell carcinoma: CALGB 90206. J. Clin. Oncol. 26 (33), 5422–5428.

Rini, B.I., Halabi, S., Rosenberg, J.E., Stadler, W.M., Vaena, D.A., Archer, L., et al., 2010. Phase III trial of bevacizumab plus interferon alfa versus interferon alfa monotherapy in patients with metastatic renal cell carcinoma: final results of CALGB 90206. J. Clin. Oncol. 28 (13), 2137–2143.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3 (6), 1101–1108.

Sultmann, H., von Heydebreck, A., Huber, W., Kuner, R., Buness, A., Vogt, M., et al., 2005. Gene expression in kidney cancer is associated with cytogenetic abnormalities, metastasis formation, and patient survival. Clin. Cancer Res. 11 (2 Pt 1), 646–655.

Takahashi, M., Rhodes, D.R., Furge, K.A., Kanayama, H., Kagawa, S., Haab, B.B., et al., 2001. Gene expression profiling of clear cell renal cell carcinoma: gene identification and prognostic classification. Proc. Natl. Acad. Sci. U. S. A. 98 (17), 9754–9759.

Tibshirani, R., 1997. The lasso method for variable selection in the Cox model. Stat. Med. 16 (4), 385–395.

Tsavachidou-Fenner, D., Tannir, N., Tamboli, P., Liu, W., Petillo, D., Teh, B., et al., 2010. Gene and protein expression markers of response to combined antiangiogenic and epidermal growth factor targeted therapy in renal cell carcinoma. Ann. Oncol. 21 (8), 1599–1606.

Tun, H.W., Marlow, L.A., von Roemeling, C.A., Cooper, S.J., Kreinest, P., Wu, K., et al., 2010. Pathway signature and cellular differentiation in clear cell renal cell carcinoma. PLoS One 5 (5), e10696.

Varela, I., Tarpey, P., Raine, K., Huang, D., Ong, C.K., Stephens, P., et al., 2011. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature 469 (7331), 539–542.

Vasselli, J.R., Shih, J.H., Iyengar, S.R., Maranchie, J., Riss, J., Worrell, R., et al., 2003. Predicting survival in patients with metastatic kidney cancer by gene-expression profiling in the primary tumor. Proc. Natl. Acad. Sci. U. S. A. 100 (12), 6958–6963.

Zhao, H., Ljungberg, B., Grankvist, K., Rasmuson, T., Tibshirani, R., Brooks, J.D., 2006. Gene expression profiling predicts survival in conventional renal cell carcinoma. PLoS Med. 3 (1), e13.