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Kidney Cancer

Systematic Evaluation of the Prognostic Impact and Intratumour Heterogeneity of Clear Cell Renal Cell Carcinoma Biomarkers

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Abstract

Background: Candidate biomarkers have been identified for clear cell renal cell carcinoma (ccRCC) patients, but most have not been validated.

Objective: To validate published ccRCC prognostic biomarkers in an independent patient cohort and to assess intratumour heterogeneity (ITH) of the most promising markers to guide biomarker optimisation.

Design, setting, and participants: Cancer-specific survival (CSS) for each of 28 identified genetic or transcriptomic biomarkers was assessed in 350 ccRCC patients. ITH was interrogated in a multiregion biopsy data set of 10 ccRCCs.

Outcome measurements and statistical analysis: Biomarker association with CSS was analysed by univariate and multivariate analyses.

Results and limitations: A total of 17 of 28 biomarkers (TP53 mutations; amplifications of chromosomes 8q, 12, 20q11.21q13.32, and 20 and deletions of 4p, 9p, 9p21.3p24.1, and 22q; low EDNRB and TSPAN7 expression and six gene expression signatures) were validated as predictors of poor CSS in univariate analysis. Tumour stage and the ccB expression signature were the only independent predictors in multivariate analysis. ITH of the ccB signature was identified in 8 of 10 tumours. Several genetic alterations that were significant in univariate analysis were enriched, and chromosomal instability indices were increased in samples expressing the ccB signature. The study may be underpowered to validate low-prevalence biomarkers.

Conclusions: The ccB signature was the only independent prognostic biomarker. Enrichment of multiple poor prognosis genetic alterations in ccB samples indicated that several events may be required to establish this aggressive phenotype, catalysed in some tumours by chromosomal instability. Multiregion assessment may improve the precision of this biomarker.

Patient summary: We evaluated the ability of published biomarkers to predict the survival of patients with clear cell kidney cancer in an independent patient cohort. Only one molecular test adds prognostic information to routine clinical assessments. This marker showed good and poor prognosis results within most individual cancers. Future biomarkers need to consider variation within tumours to improve accuracy.

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1. Introduction

The clinical behaviour of clear cell renal cell carcinomas (ccRCCs) is highly variable, ranging from slow-growing localised tumours to aggressive metastatic disease. Thus, prognostic markers are important to guide therapeutic intervention and follow-up strategies. Prognostic markers in routine clinical use include tumour stage and grade and prognostic models and nomograms that can also incorporate necrosis, blood tests such as lactate dehydrogenase, haemoglobin, platelets, and calcium levels, prior nephrectomy, symptoms, and performance status [1–6]. However, the accuracy of predictions remains limited for individual patients.

Molecular ccRCC characteristics including genetic alterations and gene expression profiles have been identified as potential novel prognostic biomarkers, but most of these have not been independently validated. Even those that have been validated have not entered clinical practice. Neither have these biomarkers been compared with each other to identify lead candidates for further development.

The analysis of multiple tumour regions from individual ccRCCs recently identified substantial intratumour heterogeneity (ITH). Spatially separated subclones harbouring distinct driver mutations and somatic copy number aberrations (SCNAS) were present within primary tumours and between primary tumours and metastases [7–9]. Phylogenetic reconstruction revealed branched evolution, demonstrating that multiple subclones were evolving simultaneously within individual tumours. Assessment of a validated prognostic gene expression signature [10] showed expression of the good prognosis ccA signature or poor prognosis ccB signature in different tumour regions within the same patient [7]. Thus ITH with spatially separated subclones can lead to sampling biases that may contribute to the lack of clinically qualified biomarkers in ccRCC. Such observations raise questions regarding how biomarker discovery strategies can be improved in heterogeneous tumours.

We identified genetic and transcriptomic prognostic biomarkers through a literature search to independently validate them in The Cancer Genome Atlas (TCGA) consortium cohort of 350 ccRCC patients [11]. Independent predictors of cancer-specific survival (CSS) were identified in multivariate analysis, and the impact of ITH was assessed.

2. Methods

2.1. Literature search

Published genetic or transcriptomic prognostic biomarkers for RCC patients were identified in PubMed and Google Scholar. Keywords included biomarker, prognosis, and renal cell carcinoma. Literature cited in review articles was also assessed. Publications had to be in the English language. Studies exclusively based on non–clear cell histology were excluded. Details of publications excluded for technical reasons can be found in the Supplement.

2.2. Validation cohort

Somatic mutation (n = 417) and clinical data (n = 446) were obtained from [11]. Single nucleotide polymorphism (SNP) array (n = 450) and RNA sequencing (RNA-seq) data (n = 469) for the same cohort were downloaded (https://tcga-data.nci.nih.gov/tcga/) on March 14, 2012, and September 18, 2012, respectively. The molecular and clinical data for our analysis were available for 350 of these patients. We used our previously published multiregion gene expression data sets GSE31610 and GSE3000 [7,8] for the assessment of ITH (data sets available at http://www.ncbi.nlm.nih.gov/geo/). Data processing is described in the Supplement.

2.3. Statistical analysis

CSS was assessed by the Kaplan-Meier method from the initial pathologic diagnosis to death with tumour as the end point. Statistical significance was assessed with the log-rank test. Hazard ratios (HRs) were calculated using univariate Cox regression analysis. Competing risk analysis was performed using death with tumour as the end point and death without tumour as the competing risk event. Variables with p ≤ 0.05 were included into multivariate Cox regression analysis with backwards stepwise selection.

Differences in enrichment of genetic aberrations and genomic instability indices in ccA and ccB subgroups were assessed by the Fisher exact test and the Wilcoxon test, respectively. Details of the statistical analysis are provided in the Supplement.

3. Results

3.1. Identification of prognostic biomarkers

The literature search identified 30 publications describing RCC prognostic genetic or gene expression markers. Three multigene expression signatures with < 70% of probes mapping to genes annotated in the TCGA RNA-seq data set and one signature based on a mathematical model optimised for array expression data and not readily applicable to RNA-seq data were excluded. Overall, 28 candidate biomarkers were identified from the remaining 26 publications for validation (Table 1).

3.2. Biomarker validation by univariate analysis

The median follow-up of the validation cohort was 51 mo. Clinical/pathologic characteristics (Table 2) were similar to the RCC cohorts in which the candidate biomarkers had been identified (Supplemental Table 1). All patients had undergone nephrectomy from which the samples for molecular analysis had been taken. Higher tumour stage and grade were significantly associated with poor CSS (Table 3 and Fig. 1) as expected. Other established clinical prognostic variables such as blood test results, performance status, or necrosis were not available for all patients and were not evaluated. A total of 19 of 28 molecular biomarkers were significantly associated (p ≤ 0.05) with CSS (Table 3).

3.2.1. Somatic mutations

Mutations in five driver genes were described as potential prognostic markers [11–18], but only nonsynonymous mutations in the BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase) (BAP1) (HR: 1.94; p = 0.022) and tumour protein 53 (TP53) (HR: 5.09; p < 0.001) tumour suppressor genes were validated as predictors of poor CSS.
| Variable | Prognosis | Analysis | Cohort size (n) | Reference |
|----------|-----------|----------|----------------|-----------|
| **Somatic mutations** | | | | |
| VHL (loss of function mutations) | Poor (OS/PPS) | Sequencing | 56 | Kim et al. [12] |
| VHL (loss of function mutations) | Poor (CSS) | Sequencing | 83 | Schraml et al. [13] |
| VHL (somatic mutations) | Better (CSS/CFS) | Sequencing | 134 | Yao et al. [14] |
| FBEM1 | Better (OS) | Sequencing | 145 + 327 | Kapur et al. [15] |
| BAP1 | Poor (OS) | Sequencing | 145 + 327 | Kapur et al. [15] |
| BAP1 | Poor (CSS) | Sequencing | 188 + 421 | Hakimi et al. [16] |
| BAP1 | Poor (OS) | Sequencing | >400 | TCGA consortium [11] |
| SETD2 | Poor (CSS) | Sequencing | 188 + 421 | Hakimi et al. [16] |
| SETD2 | Poor (CFS) | Sequencing | 240 | Sato et al. [17] |
| TP53 | Poor (CSS) | Sequencing | 416 | Randhawa et al. [18] |
| **Somatic copy number variations** | | | | |
| 5q31-qter (5q focal) Amplification | Better (CSS) | Cytogenetics | 104 | Gunawan et al. [19] |
| 7q36.2 (7q focal) Amplification | Poor (CSS) | Array CGH, FISH | 53 | Sanjmyatav et al. [20] |
| 8q Amplification | Poor (CSS) | Cytogenetics | 336 | Klatte et al. [21] |
| 8q Amplification | Poor (OS) | SNP array | 85 | Monzon et al. [22] |
| 12 Amplification | Poor (RFS) | Cytogenetics | 50 | Elfving et al. [25] |
| 20q11.21q13.32 (20q focal) Amplification | Poor (CSS) | Array CGH, FISH | 53 | Sanjmyatav et al. [20] |
| 20 Amplification | Poor (RFS) | Cytogenetics | 50 | Elfving et al. [25] |
| 3p Deletion | Better (CSS) | Cytogenetics | 246 | Klatte et al. [23] |
| 3p Deletion | Better (CSS) | Cytogenetics | 288 | Kroeger et al. [24] |
| 4p Deletion | Poor (CSS) | Cytogenetics | 246 | Klatte et al. [23] |
| 8p Deletion | Poor (RFS) | Cytogenetics | 50 | Elfving et al. [25] |
| 9p21.3p24.1 (9p focal) Deletion | Poor (CSS) | CGH, FISH | 53 | Sanjmyatav et al. [20] |
| 9p Deletion | Poor (CSS) | Cytogenetics | 246 | Klatte et al. [23] |
| 9p Deletion | Poor (CSS/RFS) | Cytogenetics, FISH | 703 | La Rochelle et al. [26] |
| 9p Deletion | Poor (RPS) | CGH | 37 | Moch et al. [27] |
| 9p Deletion | Poor (CSS) | FISH | 73 | Brunelli et al. [28] |
| 14q Deletion | Poor (CSS) | Cytogenetics | 246 | Klatte et al. [23] |
| 14q Deletion | Poor (CSS) | Cytogenetics | 288 | Kroeger et al. [24] |
| 14q Deletion | Poor (OS/RFS) | SNP array | 85 | Monzon et al. [22] |
| 19 Deletion | Poor (CSS) | Cytogenetics | 131 | Antonelli et al. [29] |
| 22 Deletion | Poor (CSS) | Cytogenetics | 131 | Antonelli et al. [29] |
| **Gene expression analysis** | | | | |
| CD31, EDNRB, and TSPAN7 expression levels | Higher expression levels of each are better (CSS) | mRNA arrays | 24 | Wuttig et al. [30] |
| Aggressive and nonaggressive ccRCCs classified using 35 genes (26 [74%] genes assessed in current study) | Aggressive worse than nonaggressive (CSS) | mRNA arrays | 66 | Kosari et al. [31] |
| Two gene expression clusters classified using 259 genes (220 [85%] genes assessed in current study) | Cluster 2 worse than cluster 1 (CSS) | mRNA arrays | 177 | Zhao et al. [33] |
| Indolent and aggressive ccRCC classified using 44 genes (36 [82%] genes assessed in current study) | Aggressive worse than indolent | cDNA arrays | 38 | Lane et al. [32] |
(Fig. 1 and Table 3). CSS was not significantly different for patients with nonsynonymous mutations in polybromo 1 (PBRM1), SET domain containing 2 (SETD2), or von Hippel-Lindau tumour suppressor, E3 ubiquitin protein ligase (VHL) (Supplemental Fig. 1). Restricting the analysis to VHL loss-of-function mutations (frameshift and nonsense mutations) in accordance with Kim et al. [12] and Schraml et al. [13] or to stage I–III cases only [14] did not change the results.

### 3.2.2. Somatic copy number alterations

Four focal SCNAs [19,20], six arm-level alterations [21–28], and four whole chromosome alterations [25,29] have been identified as candidate biomarkers. Several of these SCNAs have been identified by cytogenetic and other low-resolution analyses. Copy number profiles generated from high-resolution SNP array data from TCGA was converted into lower resolution cytoband-level data to facilitate comparison. Amplification or deletion of ≥50% of a chromosome arm or of both arms of a chromosome was considered to be equivalent to an arm-level alteration as described [11] or to a whole chromosome aberration, respectively.

Nine of 14 unique SCNAs were validated as poor prognostic markers. Chromosome 8q (Chrom8q) amplification (HR: 2.70; \( p < 0.001 \)), Chrom12 amplification (HR: 1.74; \( p = 0.034 \)), Chrom20 focal amplification (HR: 2.44; \( p < 0.001 \)), Chrom20 amplification (HR: 2.37; \( p < 0.001 \)), Chrom4p deletion (HR: 1.97; \( p = 0.019 \)), Chrom9p focal deletion (HR: 2.33; \( p < 0.001 \)), Chrom9p deletion (HR: 2.56; \( p < 0.001 \)), Chrom19 deletion (HR 3.25; \( p = 0.034 \)), and Chrom22q deletion (HR: 2.23; \( p = 0.012 \)) were significantly associated with poor CSS (Fig. 1 and Table 3). The remaining five SCNA markers failed validation (Supplemental Fig. 1).

### 3.2.3. Gene expression analysis

EDNRB and TSPAN7 gene expression above defined cut-offs [30] correlated with better CSS (HR: 0.37; \( p < 0.001 \) and HR: 0.29; \( p < 0.001 \), respectively), but CD31 overexpression was not significant. Non-negative matrix factorisation (NMF) clustering was applied for each multigene expression signature [10,31–34] to identify samples with distinct expression profiles (Supplemental Fig. 2). All prognostic

| Table 1 – (Continued) |
|------------------------|
| **Variable** | **Prognosis** | **Analysis** | **TCGA cohort (n = 350)** |
|-------------|----------------|---------------|--------------------------|
| ccA/ccB subgroup | ccB worse than ccA (CSS) | mRNA arrays | 48 * 177 Brannon et al. [10] |
| Cluster A, B, and C | Cluster A better than B and C, with C having the poorest prognosis (CSS) | mRNA arrays | 176 Beleut et al. [34] |
| TGFβ signature: scored with a panel of 157 TGFβ genes (145 [92%] genes assessed in current study) | Poor for higher expression (CSS) | mRNA arrays | 176 Bostrom et al. [35] |

CSS = cancer-specific survival; mRNA = messenger RNA; OS = overall survival; PFS = progression-free survival; SNP = single nucleotide polymorphism; TGF = tumour growth factor.

* The cohort size in this table signifies the number of cases for which follow-up data was available.

* Loss of function mutation was defined as frameshift or nonsense mutations.

| Table 2 – Patient and tumour characteristics of the validation cohort |
|------------------------|
| **Variable** | **TCGA cohort (n = 350)** |
|-------------|--------------------------|
| Age, yr | Median (IQR) 61 (52–70) |
| Gender (%) | Male 222 (63) |
| Fuhrman grade (%) | G1 4 (1) |
| Clinical stage (%) | Stage I 162 (46) |
| Primary tumour spread (%) | T1 166 (48) |
| Metastatic spread (%) | M0 293 (84) |
| ccRCC = clear cell renal cell carcinoma; TCGA = The Cancer Genome Atlas. |
Table 3 – Univariate survival analysis

| Variable                                      | No. of cases (n = 350) (%) | HR (95% CI)    | p value    |
|-----------------------------------------------|----------------------------|---------------|------------|
| **Clinical and pathologic characteristics**   |                            |               |            |
| Stage II vs stage I                           | 34 (10)                    | 4.45 (1.55–12.77) | 0.006      |
| Stage III vs stage I                          | 96 (27)                    | 7.34 (3.16–17.08) | <0.001     |
| Stage IV vs stage I                           | 58 (17)                    | 25.24 (11.26–56.71) | <0.001     |
| G3 vs G1/G2                                   | 146 (42)                   | 2.35 (1.30–4.26)  | 0.005      |
| G4 vs G1/G2                                   | 55 (16)                    | 7.43 (3.99–13.81)  | <0.001     |
| **Somatic mutations**                         |                            |               |            |
| VHL loss of function mutation                 | 86 (24.5)                  | 0.59 (0.34–1.04)  | 0.064      |
| VHL nonsyn mutation (all cases)               | 178 (51)                   | 0.80 (0.51–1.25)  | 0.323      |
| VHL nonsyn mutations (stage I–III cases)     | 155/292 (53)               | 0.95 (0.50–1.80)  | 0.873      |
| PPARG1 nonsyn mutation                        | 117 (33)                   | 0.90 (0.56–1.43)  | 0.643      |
| BAP1 nonsyn mutation                          | 37 (10.5)                  | 1.94 (1.08–3.45)  | 0.022      |
| SETD2 nonsyn mutation                         | 39 (11)                    | 1.41 (0.76–2.60)  | 0.273      |
| TP53 nonsyn mutation                          | 7 (2)                      | 5.09 (1.85–14.00)  | <0.001     |
| **Somatic copy number variations**            |                            |               |            |
| 5q focal amplification                        | 191 (54.5)                 | 0.72 (0.47–1.12)  | 0.143      |
| 7q focal amplification                        | 95 (27)                    | 1.29 (0.81–2.05)  | 0.283      |
| 8q amplification                              | 33 (9)                     | 2.70 (1.52–4.81)  | <0.001     |
| 12 amplification                              | 56 (16)                    | 1.74 (1.04–2.91)  | 0.034      |
| 20q focal amplification                       | 51 (15)                    | 2.44 (1.49–3.99)  | <0.001     |
| 20 amplification                              | 47 (13)                    | 2.37 (1.41–3.97)  | <0.001     |
| 3p deletion                                   | 318 (91)                   | 0.86 (0.41–1.79)  | 0.687      |
| 4p deletion                                   | 42 (12)                    | 1.97 (1.10–3.52)  | 0.019      |
| 8p deletion                                   | 101 (29)                   | 1.58 (0.99–2.50)  | 0.051      |
| 9p focal deletion                              | 85 (24)                    | 2.33 (1.49–3.64)  | <0.001     |
| 9p deletion                                   | 88 (25)                    | 2.56 (1.64–3.99)  | <0.001     |
| 14q deletion                                  | 140 (40)                   | 1.51 (0.97–2.35)  | 0.064      |
| 19 deletion                                   | 6 (1.7)                    | 3.25 (1.02–10.32) | 0.034      |
| 22q deletion                                  | 26 (7)                     | 2.23 (1.18–4.23)  | 0.012      |
| **Gene expression analysis**                  |                            |               |            |
| CD31 expression                               |                            |               |            |
| < median                                      | 175 (50)                   | 0.64 (0.41–1.01)  | 0.051      |
| ≥ median                                      | 175 (50)                   |               |            |
| EDNRB expression                              |                            |               |            |
| < median                                      | 175 (50)                   | 0.37 (0.23–0.59)  | <0.001     |
| ≥ median                                      | 175 (50)                   |               |            |
| TSPAN7 expression                             |                            |               |            |
| <33 percentile                                | 105 (30)                   | 0.29 (0.18–0.45)  | <0.001     |
| ≥33 percentile                                | 245 (70)                   |               |            |
| Kosari signature                              |                            |               |            |
| Nonaggressive                                 | 242 (69)                   | 2.85 (1.84–4.43)  | <0.001     |
| Aggressive                                    | 108 (31)                   |               |            |
| Zhao signature                                |                            |               |            |
| Cluster 1 (good)                              | 269 (77)                   | 5.26 (3.37–8.22)  | <0.001     |
| Cluster 2 (poor)                              | 81 (23)                    |               |            |
| Lane signature                                |                            |               |            |
| Indolent                                      | 219 (63)                   | 4.21 (2.62–6.77)  | <0.001     |
| Aggressive                                    | 131 (37)                   |               |            |
| ccA/ccB status                                |                            |               |            |
| ccA                                           | 240 (69)                   | 4.90 (3.09–7.76)  | <0.001     |
| ccB                                           | 110 (31)                   |               |            |
| Beulet signature                              |                            |               |            |
| Cluster A                                     | 127 (36)                   | 1.00 (Ref)  | 0.005      |
| Cluster B                                     | 175 (50)                   | 2.27 (1.31–3.96)  | <0.001     |
| Cluster C                                     | 48 (14)                    | 2.30 (1.13–4.66)  | <0.001     |
| TGFβn signature                               |                            |               |            |
| Low expression score                          | 175 (50)                   | 1.98 (1.23–3.16)  | 0.003      |
| High expression score                         | 175 (50)                   |               |            |

CI = confidence interval; HR = hazard ratio; nonsyn = nonsynonymous; TGF = tumour growth factor.
Fig. 1 – Kaplan-Meier survival estimates for cancer-specific survival for clinical and genetic markers: (A) tumour stage; (B) Fuhrman grade; (C) BAP1 nonsynonymous (nonsyn) mutation status; (D) TP53 nonsyn mutation status; (E) chromosome (Chrom) 8q amplification (amp) status; (F) Chrom12 amp status; (G) Chrom20q focal amp status; (H) Chrom20 amp status; (I) Chrom4p deletion (del) status; (J) Chrom9p focal del status; (K) Chrom9p del status; (L) Chrom19 del status; (M) Chrom22q del status.

WT = wild type.
gene expression signatures validated: the aggressive subgroup defined by Kosari [31] had worse CSS than the nonaggressive subgroup (HR: 2.85; \(p < 0.001\)); the Zhao [33] poor prognosis cluster 2 had worse CSS than cluster 1 (HR: 5.26; \(p < 0.001\)). The aggressive subgroup defined by Lane et al. [32] showed worse CSS than the indolent subgroup (HR: 4.21; \(p < 0.001\)); the Brannon [10] poor prognosis ccB subgroup (HR: 4.90; \(p < 0.001\)) had worse CSS than the ccA subgroup. Based on Beleut et al. [34], CSS was significantly worse for patients in the poor prognosis clusters C (HR: 2.21; \(p = 0.034\)) and B (HR: 2.46; \(p = 0.002\)) than for those in cluster A, although CSS of clusters B and C showed no significant difference. The poor-risk subgroup of Böstrom et al. [35] with a high tumour growth factor \(\beta\) (TGF-\(\beta\)) score had worse CSS than the subgroup with a low score (HR: 1.98; \(p = 0.003\)) (Fig. 2 and Table 3).

With the exception of BAP1 mutations and Chrom19 deletions, all markers that were significant in log-rank analysis were also significant in a competing risk analysis including death from causes other than cancer.

### 3.3. Identification of independent biomarkers in multivariate analysis

Chrom9p focal deletion and Chrom20 whole arm amplification were excluded because their HRs were lower than the overlapping Chrom9p arm-level deletions and Chrom20 focal amplifications. The remaining 17 biomarkers that had...
been validated in the log-rank analysis were included together with tumour stage and grade into the multivariate analysis (MVA). Tumour stage, the ccA/ccB gene expression signature, and Chrom19 deletions were the only independent predictors of CSS (Table 4 and Supplemental Table 2). After exclusion of the two markers (BAP1 mutations, Chrom19 deletions) that were not significant in the competing risk analysis, only tumour stage and the ccA/ccB gene expression signature remained significant in MVA (Table 4). Based on these results and the small number of six tumours showing Chrom19 deletions, the ccB signature was the lead candidate for further assessment.

The ccB signature was consistently associated with a worse prognosis in patients with stage I (HR > 10; \( p < 0.001 \)), stage II/III (HR: 3.03; \( p = 0.003 \)), and stage IV ccRCCs (HR: 2.15; \( p = 0.015 \)) (Supplemental Fig. 3). A total of 135 patients with stage I tumours expressing the ccA signature demonstrated particularly good outcomes with no cancer-specific deaths for >6 yr. The ccA/ccB signature was also significant in MVA when assessed together with the validated and widely used size, stage, grade, and necrosis (SSIGN) prognostic scoring system [4,36,37] (data available for a subgroup of 334 patients; Supplemental Table 3). CSS of patients whose tumours displayed the ccA or ccB signature were significantly different in three of five validated SSIGN score categories [36,37] (Supplemental Fig. 4). The ccA/ccB signature could not be compared with other clinical nomograms [1,2,5,6] because essential parameters were not available for most of the patients in the TCGA cohort. After completion of our literature search, the ClearCode34 prognostic expression signature was published that is based on the ccA/ccB signature [38]. This signature was significant in univariate analysis and together with tumour stage in MVA if the ccA/ccB signature was omitted (Supplemental Table 4). Although the HR for ClearCode34 in the MVA was lower (HR: 2.23) than that of the ccA/ccB signature (HR: 2.95), the implementation of this 34-gene signature may be easier in clinical practice than the 110-gene ccA/ccB signature.

### 3.4. Molecular drivers of the ccB subgroup

We next investigated whether the ccB expression signature might reflect the transcriptomic impact of the poor-risk genetic alterations that were significant in log-rank analysis but failed in the multivariate analysis. Seven of nine poor prognosis genetic alterations (BAP1 and TP53 mutations; Chrom8q, Chrom12, and Chrom20q focal amplifications; Chrom9p and Chrom22q deletions) were significantly enriched \( (p < 0.05) \) in the ccB subgroup (Fig. 3). Overall, 72% of the ccB samples showed at least one of these seven aberrations compared with only 30% of ccA samples (Fig. 4A). Both the maximum and the median number of these aberrations per sample were higher in the ccB group than in the ccA group (Fig. 4A and 4B). In contrast, only two of the eight candidate genetic markers that had failed univariate validation were enriched in ccB samples (Supplemental Fig. 5), and the median number of these aberrations between ccA and ccB samples was not statistically different (Fig. 4C and 4D).

Chromosomal instability fosters the acquisition of SCNAs and has been associated with poor prognosis in several cancers [39]. To reveal whether enrichment of chromosomal aberrations in ccB was a result of increased chromosomal instability, we calculated the weighted Genomic Instability Index (wGII), which is a measure of overall copy number aberrations (wGII > 0.2 is considered unstable [40]). The ccB samples had significantly higher wGIs compared with ccA samples \( (p < 0.001; \text{Fig. 4E}) \). Based on these results, it appears possible that the aggressive ccB phenotype is partially driven by several poor prognosis SCNAs co-occurring within these samples, permitted by a cancer genomic background of elevated chromosomal instability.

### 3.5. Intratumour heterogeneity of the ccA/ccB signature

We previously found that the ccA and the ccB signatures were present simultaneously within an individual ccRCC [7]. To investigate whether this signature commonly displays ITH, we reanalysed our published gene expression data of 63 tumour regions from 10 stage II–IV ccRCCs [7,8] (Supplemental Fig. 6) and mapped the results onto the phylogenetic trees previously published for these tumours [8] (Fig. 5). Only two tumours homogeneously expressed the ccA signature; the other eight tumours were heterogeneous with ccA and ccB components detectable, suggesting the need to sample multiple tumour regions to reliably detect poor prognostic clones.

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**Table 4 – Multivariate survival analysis**

| Variable                        | Including BAP1 mutations and chromosome 19 deletion | Excluding BAP1 mutations and chromosome 19 deletion |
|---------------------------------|-----------------------------------------------------|-----------------------------------------------------|
|                                 | Hazard ratio (95% CI) | p value | Hazard ratio (95% CI) | p value |
| Tumour stage                    |                       |         |                       |         |
| Stage I                         | 1.00 (Ref)            |         | 1.00 (Ref)            |         |
| Stage II                        | 3.48 (1.20–10.06)     | 0.022   | 3.40 (1.18–9.82)      | 0.024   |
| Stage III                       | 4.61 (1.93–11.00)     | <0.001  | 4.86 (2.05–11.55)     | <0.001  |
| Stage IV                        | 18.01 (7.89–41.12)    | <0.001  | 17.77 (7.79–40.53)    | <0.001  |
| Chromosome 19 deletion          | 4.18 (1.27–13.69)     | 0.018   | –                     | –       |
| ccA status                      | 1.00 (Ref)            |         | 1.00 (Ref)            |         |
| ccB status                      | 2.99 (1.87–4.80)      | <0.001  | 2.95 (1.84–4.72)      | <0.001  |

CI = confidence interval.
4. Discussion

A total of 17 of the 28 published genetic and transcriptomic prognostic ccRCC markers were validated in log-rank and competing risk analysis as predictors of CSS in this independent validation cohort. Of those, only the ccB gene expression signature was significant in MVA. Tumour stage was the only other independent predictor of CSS in MVA. Importantly, the ccA signature identified patients with stage I ccRCCs who had an excellent prognosis with no cancer-specific deaths over >6 yr of follow-up. The ccA/ccB signature was also significant in MVA with the established SSIGN prediction model, demonstrating that this molecular marker can add additional information to one of the best currently available predictors based on clinical and pathologic information. Thus the ccA/ccB signature could refine personalised follow-up strategies or stratification into adjuvant therapy trials. The novel ClearCode34

Fig. 3 – Heat map showing consensus non-negative matrix factorisation clustering analysis based on gene expression data of 103 ccA/ccB signature genes. Patient assignment to ccA and ccB prognostic subgroups is indicated by coloured bars at the top of the heat map. Coloured bars below the heat map depict the presence of poor prognosis genetic aberrations. The bar chart at the bottom of the figure represents the number of these genetic aberrations per patient. OR = odds ratio.
signature is based on the ccA/ccB signature but can be assessed from 34 instead of 110 genes. The performance of this new marker was slightly inferior, but it may nevertheless be valuable because clinical adoption may be easier.

Previous work revealed that genes overexpressed in samples with the ccA signature are enriched for genes implicated in angiogenesis and fatty acid, organic acid, and pyruvate metabolism. Genes overexpressed in samples displaying the ccB signature are enriched for cell differentiation, epithelial to mesenchymal transition, mitotic cell cycle, response to wounding, and TGF-β and Wnt signalling genes [10]. We further revealed that seven of nine specific genetic alterations that were validated in univariate analysis were enriched in ccB samples with 72% of samples harbouring at least one and up to six of these. These genetic changes were only found in 30% of the ccA samples with a maximum of four aberrations per sample. Thus the ccB signature may reflect the transcriptomic impact of these poor prognosis alterations, but more than one alteration may be necessary to establish this phenotype, and as yet unknown alterations are also likely to contribute. Arguably, prognostic markers are of limited clinical utility in ccRCC due to the current absence of effective adjuvant strategies. However, further study of the interplay of these genetic aberrations and the pathways deregulated in the ccB signature are clearly necessary to reveal the mechanisms and biologic implications of the ccB phenotype. Such insights could eventually foster the development of specific therapeutic approaches for poor prognosis ccRCC.

Chromosomal instability indices (wGII) were higher in ccB than in ccA samples, suggesting that chromosomal instability may catalyse the evolution of the ccB phenotype.

Fig. 4 – (A) Comparison of the number of poor prognosis genetic aberrations per sample between ccA and ccB subgroups. Only aberrations that are enriched in the ccB subgroup were considered. (B) Box and whisker plot comparing median number of poor prognosis genetic aberrations between samples assigned to the ccA and the ccB group. (Wilcoxon test; $p < 0.001$). (C) Comparison of the number of number of genetic aberrations that did not pass univariate validation per sample between ccA and ccB subgroups. (D) Box plot and whisker plot showing the median number of genetic aberrations that did not pass univariate validation between ccA and ccB subgroups (Wilcoxon test; $p = 0.138$). (E) Box plot and whisker plot comparing weighted Genomic Instability Index (wGII) between ccA and ccB subgroups. wGII ≥0.2 is considered genomically unstable.
by providing the permissive heterogeneous genomic background from which these SCNAs can be selected. These results are hypothesis generating and will require further study.

Evaluation of the ccA/ccB signature across multiple tumour regions from each of 10 stage II–IV ccRCCs demonstrated heterogeneous expression patterns with ccA and ccB signatures coexisting in 8 of 10 cases. ITH with spatial separation of subclones that may harbour distinct transcriptomic profiles demonstrates that single biopsies are unlikely to reveal a complete picture of the landscape of even the best current binary classification ccRCC biomarkers.

These data suggest some interesting avenues for research. Despite ITH, the ccB signature outperforms every other candidate biomarker in this analysis. It is currently unknown whether a tumour with a small ccB component has a similarly poor prognosis to an identical size tumour dominated by the ccB signature. If the absolute size of the poor-risk clone, irrespective of the entire tumour population, is the most critical parameter, then ITH may be less problematic in small tumours because the chance of analytical techniques sampling the high-risk cell population would be high. However, detection of a poor-risk ccB clone in larger tumours may be more difficult unless the entire tumour is sampled or dominated by the ccB signature. These

Fig. 5 – Heterogeneity analysis of ccA/ccB expression profiles. The ccA or ccB profiles detected by consensus non-negative matrix factorisation clustering in a multiregion analysis data set from 10 clear cell renal cell carcinomas were mapped onto the phylogenetic trees of these tumours (adapted with permission from Nature Publishing Group [8]). Regional gene expression signatures were assigned to the dominant clones detected within the region. The minority clones detected in some regions in the original publication were omitted.
5. Conclusions

Taken together, this study suggests that the ccA/ccB gene expression signature outperforms other transcriptomic and genetic biomarkers for the prediction of ccRCC CSS and that it adds prognostic information to tumour stage and to the SSIGN prognostic model. This signature could be particularly relevant for the profiling of stage I ccRCCs where the detection of the ccA signature was associated with an excellent prognosis. Stage I ccA tumours may only require minimal follow-up, whereas ccB tumours may benefit from more stringent surveillance and may be good candidates for adjuvant therapy trials. Multiregion profiling of larger cohorts could define how to integrate heterogeneity assessments into biomarker predictions and further improve the accuracy of the ccA/ccB signature.

Author contributions: Charles Swanton and Paul A Bates had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data: Gulati, Gerlinger, Bates, Swanton.

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Appendix A. Supplementary data

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