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Platinum Priority – Brief Correspondence

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A Multigene Assay Identifying Distinct Prognostic Subtypes of Clear Cell Renal Cell Carcinoma with Differential Response to Tyrosine Kinase Inhibition

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Abstract

Patients with clear cell renal cell carcinoma (ccRCC) have divergent survival outcomes and therapeutic responses, which may be determined by underlying molecular diversity. We aimed to develop a practical molecular assay that can identify subtypes with differential prognosis and response to targeted therapy. Whole-genome expression analysis of formalin-fixed paraffin-embedded (FFPE) material from 55 ccRCC patients was performed and two molecular subtypes with differential clinical outcomes were identified by hierarchical clustering. An eight-gene quantitative polymerase chain reaction assay for classification into two subtypes was developed for FFPE material. The primary objective was to assess assay performance by correlating ccRCC prognostic subtypes to cancer-specific survival (CSS) and, for patients receiving targeted therapy, radiologic response. In three validation cohorts, patients could be distinguished into prognostic subtypes with differential CSS (Singapore General Hospital FFPE cohort: $n = 224$; $p = 1.48 \times 10^{-8}$; the Cancer Genome Atlas RNA-Sequencing cohort: $n = 419$; $p = 3.06 \times 10^{-7}$; Van Andel Research Institute microarray cohort: $n = 174$; $p = 0.00743$). For 48 patients receiving tyrosine kinase inhibitor (TKI) treatment, the prognostic classification was associated with radiologic response to treatment ($p = 5.96 \times 10^{-4}$) and prolonged survival on TKI treatment ($p = 0.019$). The multigene assay can classify ccRCCs into clinical prognostic subtypes, which may be predictive of response in patients receiving TKI therapy.

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About 30% of localized clear cell renal cell carcinomas (ccRCC) relapse after curative surgery [1]. While tumor stage at presentation remains the most reliable predictor of clinical course of disease after surgery, survival outcomes are heterogeneous within each staging group [2]. For advanced ccRCC, survival and treatment response are similarly variable, even in the era of targeted therapy [3,4]. Extensive molecular characterization of ccRCC suggests that subtypes exist with distinct survival advantages [5,6]. In such a heterogeneous disease setting, discovering reliable biomarkers that can improve prognostic determination and identify patients likely to benefit from treatment is of high

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priority. Consideration of molecular features of ccRCCs in existing risk-stratification models for predicting survival after treatment may enhance clinical decision making [7,8]. In this study, we developed a practical expression-based assay with utility in formalin-fixed paraffin-embedded (FFPE) material that assigns biologic subtypes of ccRCC, characterized by differential prognosis and treatment response.

The study was conducted retrospectively with a cohort of 279 ccRCC patients who underwent surgery at Singapore General Hospital (SGH) between 1999 and 2012. Patient characteristics are described in Supplemental Table 1 and the clinical data collection process in the Supplement. The overall analysis pipeline is described in Supplemental Figure 1. Initially, to identify relevant biologic subtypes of ccRCC, RNA was extracted from a set of 55 FFPE samples (SGH-55) and processed for whole-genome expression analysis by Whole Genome (WG)-DASL (Illumina, San Diego, CA, USA) (Supplement 1).

Hierarchical clustering based on expression of 3740 transcripts measured by WG-DASL partitioned samples from SGH-55 into two main groups (Fig. 1A; Supplemental Table 2). Kaplan-Meier analysis showed that the two biologically determined subtypes of ccRCC differed in cancer-specific survival (CSS) (hazard ratio [HR]: 8.70; 95% confidence interval [CI], 1.69–44.89; $p = 0.00185$) (Fig. 1B). The subtypes were also associated with relevant clinicopathologic features: tumor grade, stage, and size (Supplemental Fig. 2). Potential prognostic genes were selected from gene features that were significantly different between the two subtypes (Supplement; Supplemental Table 3).

Quantitative polymerase chain reaction (qPCR) assays were designed for measuring expression in FFPE tissue; expression data for potential prognostic and normalization genes for SGH-55 were collected. Processing of qPCR expression data, selection of prognostic genes, and development of the prediction model are described in the Supplement. A model assigning prognostic subtype was developed based on the combination of qPCR expression values of eight genes: chemokine (C-X-C motif) ligand 5 (*CXCL5*), ephrin A5 (*EFNA5*), endomucin (*EMCN*), laminin beta3 (*LAMB3*), plasminogen (*PLG*), preferentially expressed antigen in melanoma (*PRAME*), retinoic acid receptor responder (tazarotene induced) 1 (*RARRES1*), and solute carrier family 6 (neutral amino acid transporter), member 19 (*SLC6A19*).

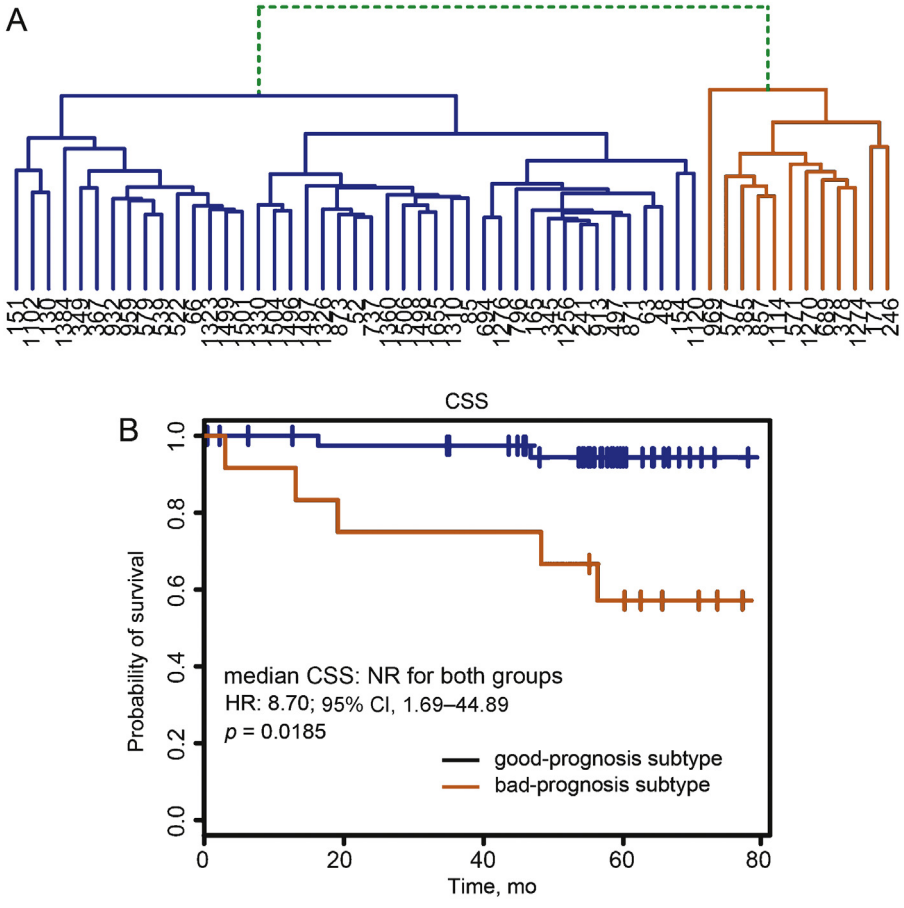

Fig. 1 – Hierarchical clustering analysis of 55 clear cell renal cell carcinomas (ccRCC) based on DASL expression data identifies two prognostic subtypes. (A) Cluster dendrogram of 55 ccRCC samples grouped by expression of 3740 genes measured by DASL analysis. Two main groups are formed ($n_1 = 43$ and $n_2 = 12$), denoted by blue branches and orange branches in the dendrogram. (B) Kaplan-Meier curves of cancer-specific survival (CSS) for two prognostic subtypes generated by hierarchical clustering. Survival in the good-prognosis subtype is significantly better than in the poor-prognosis group (log-rank test $p = 0.0185$).
CI = confidence interval; HR = hazard ratio; NR = not reached.

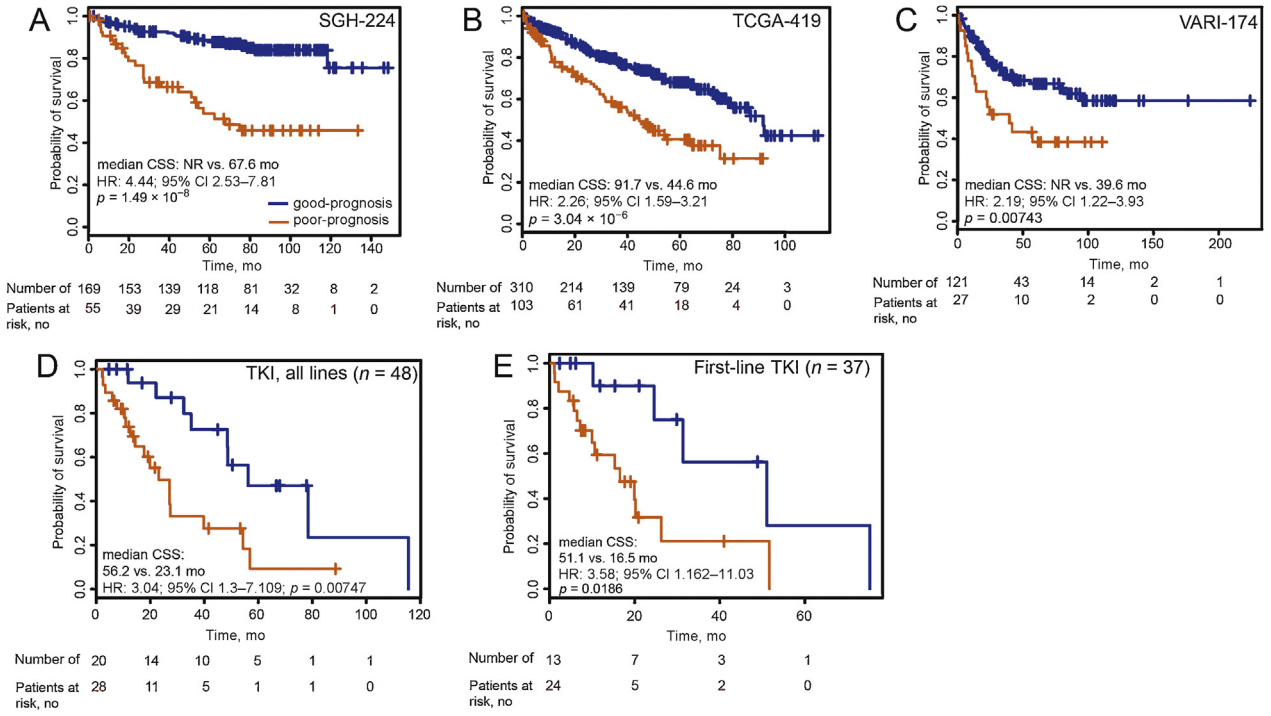

Fig. 2 – Validation of an eight-gene, prognosis subtype-classification algorithm for clear cell renal cell carcinomas (ccRCC), including utility in predicting survival in patients with metastatic ccRCC who received tyrosine kinase inhibitor (TKI) therapy. (A–C) Survival analysis by Kaplan-Meier method for ccRCC patients classified into good- and poor-prognosis subtypes based on expression of eight genes. A difference is observed in cancer-specific survival (CSS) between two prognosis subtypes. (D–E) Survival analysis for TKI-receiving patients similarly classified into prognosis groups. The *p* values are derived from log-rank tests. (A) Prognostic subtype assignment for Singapore General Hospital (SGH)-224 validation cohort (*n* = 224) based on quantitative polymerase chain reaction gene expression measurement in formalin-fixed paraffin-embedded tumors. (B) Prognostic subtype assignment for the Cancer Genome Atlas (TCGA)-419 validation cohort (*n* = 419) by classification algorithm applied to RNA-sequencing expression data. It should be noted that the TCGA dataset is enriched in patients with higher-grade disease with an overall poor survival outlook, with only five samples classified as histologic grade 1 tumors. (C) Prognostic subtype assignment for Van Andel Research Institute (VARI)-174 validation cohort based on Affymetrix microarray expression data (Affymetrix, Santa Clara, CA, USA). (D) CSS of patients with metastatic ccRCC receiving TKI therapy in first-, second-, and third-line settings. (E) CSS of patients with metastatic ccRCC receiving TKI therapy in the first-line setting.
CI = confidence interval; HR = hazard ratio; NR = median survival time not reached.

The performance of the eight-gene prognostic assay was validated in an independent cohort of 224 FFPE ccRCC samples (SGH-224) for which qPCR expression data were processed. There was a significant CSS difference between good- and poor-prognosis subtypes (HR: 4.44; 95% CI, 2.53–7.81; $p = 1.49 \times 10^{-8}$) (Fig. 2A). In a multivariate analysis adjusting for standard clinicopathologic parameters, prognostic class assignment remained significantly correlated with CSS (Supplemental Table 8).

To demonstrate its utility and validate it in a multicenter, multiplatform setting, the prognostic algorithm was applied to two other datasets (Supplement). For the Cancer Genome Atlas (TCGA)-419 dataset, Kaplan-Meier analysis confirmed that CSS was significantly different between prognostic subtypes (HR: 2.26; 95% CI, 1.59–3.21; $p = 3.04 \times 10^{-6}$) (Fig. 2B). Similarly, for the Van Andel Reasearch Institute (VARI)-174 dataset, prognostic subtypes had significantly different CSS outcomes (HR: 2.19; CI, 1.22–3.93; $p = 0.00743$) (Fig. 2C).

For a subset of 48 patients from the SGH-224 cohort who had metastatic RCC and received tyrosine kinase inhibitor (TKI) treatment in a first-, second-, or third-line setting, a similar prognostic classification was done. Characteristics of TKI-receiving patients are presented in Supplemental Table 9. Univariate logistic regression analysis with clinical benefit as a categorical variable and prognostic class assignment showed a significant correlation between the two (odds ratio: 0.429; $p = 5.96 \times 10^{-4}$) (Supplemental Table 10). Among patients receiving TKI therapy, survival was similarly predicted to be longer for patients in the good-prognosis subtype (HR: 3.04; CI, 1.3–7.109; $p = 0.00747$) (Fig. 2D). When analysis was restricted to patients receiving TKI in first-line setting and survival time from initiation of TKI treatment was considered, patients of good-prognosis subtype survived longer (HR: 3.58; CI, 1.162–11.03; $p = 0.0186$) (Fig. 2E). The eight-gene assay continued to predict survival differences after patient stratification by Memorial Sloan-Kettering Cancer Center risk criteria ($p = 0.0276$) [7].

We have developed a practical molecular assay capable of classifying ccRCC patients into prognostic subtypes that manifest the underlying biologic heterogeneity of ccRCC. Subtypes of ccRCCs were first identified in 2001 using gene-expression profiling of 29 ccRCCs [9]. While useful for understanding biologic variations among ccRCCs, the implementation of such a subtype signature is practically limited due to the cost and limited availability of fresh-frozen tissue.

An additional utility of our eight-gene predictor is the ability to predict benefit from TKI therapy. Previous attempts to identify predictive biomarkers for TKI therapy for ccRCC, which is the most common therapy administered, have included serum- and tissue-based markers, but still require validation in prospective setting [10].

The eight genes in the prognostic assay—*CXCL5*, *EFNA5*, *EMCN*, *LAMB3*, *PLG*, *PRAME*, *RARRES1*, and *SLC6A19*—represent genes from the chemokine signaling, migration and invasion, angiogenesis, growth-factor signaling, extracellular matrix-interacting, retinoic-acid signaling, and transporter families (known functions listed in Supplemental Table 11). The unbiased selection method starting with WG expression analysis likely accounts for the wide variety of cellular functions encompassed in the prognostic gene set.

The limitations of this study are its retrospective design and the relatively limited number of subjects for TKI-response prediction analysis. External validation in prospective trials will be crucial to determine clinical value. The utility of the assay in abundantly available FFPE material does greatly broaden the scope for rapid validation, particularly in legacy material from previously conducted phase 3 trials. A feature of this study is the validation of the expression signatures in cohorts from different expression platforms, derived from several centers and geographic locations. Prognostic signatures ideally should be considered alongside optimal clinical predictors of outcome, such as the Mayo Clinical Stage, Size, Grade and Necrosis [8] and the International Metastatic Renal Cell Carcinoma Database Consortium model. Future systematic studies will be important to address this.

In conclusion, we have designed a practical FFPE gene expression assay for ccRCC classifying tumors into prognostic subtypes, with potential implications for therapeutic response.

Author contributions: Min-Han Tan had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data: Choudhury, Wei, M.-H. Tan.
Drafting of the manuscript: Choudhury, M.-H. Tan.
Critical revision of the manuscript for important intellectual content: Choudhury, Wei, Chua, L.G. Ng, H.S. Tan, Koh, Thike, Poon, Q.S. Ng, Toh, Kanesvaran, P.H. Tan, M.-H. Tan.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eururo.2014.06.041.

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