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Ultrasensitive Circulating Tumor DNA Pilot Study Distinguishes Complete Response and Partial Response With Immunotherapy in Patients With Metastatic Renal Cell Carcinoma

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abstract

PURPOSE Circulating tumor DNA (ctDNA) has been validated across multiple indications in the adjuvant and surveillance settings. We evaluated whether targeted digital sequencing (TARDIS) may distinguish a partial response (PR) from a complete response (CR) among patients with metastatic renal cell carcinoma (mRCC) receiving immune checkpoint inhibitor (ICI) therapy.

MATERIALS AND METHODS Eligible patients had mRCC that yielded a PR or CR to ICI therapy. Peripheral blood was obtained at a single time point for ctDNA analysis. TARDIS was used for quantification of average variant allele fractions (VAFs). Our primary objective was to determine the association between VAFs and depth of response (PR *v* CR). A secondary objective was to determine whether VAFs were associated with disease progression.

RESULTS Twelve patients were analyzed, nine of whom achieved a PR (75%). Patients received either nivolumab monotherapy (50%) or nivolumab plus ipilimumab (50%). ctDNA analysis incorporated an average of 30 patient-specific mutations (range, 19-35); average coverage depth was 103,342 reads per target. TARDIS quantified a significant difference in VAFs between PR and CR (median, 0.181% [IQR, 0.077%-0.420%] *v* 0.007% [IQR, 0.0%-0.028%], respectively [$P = .014$]). Of the 12 patients in the series, six patients demonstrated radiographic progression subsequent to ctDNA assessment. Patients who progressed on subsequent scans had significantly higher ctDNA than those who maintained their response (median, 0.362% [IQR, 0.181%-2.71%] *v* 0.033% [IQR, 0.007%-0.077%], respectively [$P = .026$]).

CONCLUSION In this pilot study, TARDIS accurately differentiated PR from CR among patients with mRCC receiving immunotherapy, and also prospectively identified patients at risk for subsequent progression. Given these findings, we envision subsequent studies that validate these results and investigate the utility of this assay to discern appropriate candidates for discontinuation of immunotherapy.

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INTRODUCTION

Immune checkpoint inhibitors (ICIs) have become a cornerstone of frontline therapy for metastatic renal cell carcinoma (mRCC), either with dual ICI therapy or in combination with targeted therapy now representing the standard of care.[1-4] An emerging challenge is whether treatment with ICIs can be withdrawn among those patients who achieve a durable response to therapy. The rate of complete response (CR) varies across malignancies with ICI therapy, ranging from 19% to 22% in advanced melanoma and 1% to 3% in advanced non–small-cell lung cancer.[5-8] In registrational mRCC trials, frontline ICI-based therapy demonstrated considerably higher rates of partial response (32%) and CR (9%) compared with second-line therapy (24% and 1%, respectively).[4,9] Beyond these subsets lies an additional group of patients who achieve a durable response to therapy.

Analysis of circulating tumor DNA (ctDNA) is a non-invasive approach to detect and quantify tumor-associated mutations in the plasma of patients with cancer. Current methods for ctDNA analysis can be classified into one of two categories: tumor-agnostic and tumor-informed.[10] The former uses standardized, fixed panels that target known somatic mutations without sequencing patient-specific formalin-fixed paraffin-embedded (FFPE) tumor samples to identify alterations. The latter uses next-generation genomic

ASSOCIATED CONTENT

Appendix

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

Previous applications of circulating tumor DNA (ctDNA) assays in patients with renal cell carcinoma (RCC) have yielded low sensitivity and specificity. Targeted digital sequencing (TARDIS) is a novel tumor-informed ctDNA assay capable of using up to 100 baseline mutations. We sought to determine the association between variant allele fractions (VAFs) and depth of response to immunotherapy in patients with metastatic RCC.

Knowledge Generated

TARDIS achieved a significant difference in VAF concentrations between partial responders and complete responders ($P = .018$). Additionally, TARDIS reached a significant difference in VAF concentrations between those with sustained radiographic response and those who progressed on subsequent imaging ($P = .026$).

Relevance

To our knowledge, this pilot study represents the first ctDNA assay to effectively discriminate between partial and complete responses to immunotherapy in RCC. These data indicate that TARDIS may be an efficacious platform for detecting molecular residual disease and relapse in metastatic RCC.

sequencing of patient FFPE tumor tissue to identify patient-specific alterations for the development of bespoke ctDNA panels for subsequent blood surveillance.

One of the major challenges with ctDNA analysis has been achieving sensitivity and quantitative precision in the setting of low ctDNA concentrations, despite limited blood volumes, to effectively prognosticate clinical outcomes.[11-14] To this end, we have developed a novel bespoke platform (targeted digital sequencing [TARDIS]) that ascertains up to 100 baseline mutations in patient FFPE tumor tissue to create a personalized assay for blood sample analysis.[15] Preliminary results from 33 patients with early and locally advanced breast cancer identified pretreatment ctDNA in 100% of patients at a mean concentration of 0.11%. After neoadjuvant chemotherapy, ctDNA dropped to 0.017% and 0.003% in patients with residual disease versus pathologic CR, respectively.[16] In the current study, we hypothesized that if TARDIS is capable of detecting ctDNA in patients with mRCC receiving ICI therapy, then it could discriminate PRs from CRs.

MATERIALS AND METHODS

Patient Selection and Sample Acquisition

Between July 1, 2020, and October 1, 2020, patients diagnosed with mRCC by standard criteria were prospectively identified at a single center using an institutional database.[17] Patients were eligible if they achieved a PR or CR to a commercially available ICI (PD-1 and/or CTLA-4 inhibitor). Enrollment was open to patients across all renal cell carcinoma (RCC) histologic subtypes and lines of therapy. Whole blood was collected at a single time point in two 10-mL Cell-Free DNA BCT tubes (Streck, La Vista, NE) from eligible patients. Demographic data were collected for each patient.

The protocol was approved by the institutional scientific review committee, data safety monitoring board, and the institutional review board at the City of Hope Comprehensive Cancer Center. The study conformed to the amended Declaration of Helsinki and the International Conference on Harmonisation Guidelines.

Tumor Genomic Sequencing and Analysis

For whole-genome sequencing (WGS), DNA was extracted from FFPE tumor samples using a truXTRAC Total Kit (Covaris, Inc, Woburn, MA) or from blood as a source of normal DNA using QIAcube automated sample preparation system (QIAGEN USA, Chatsworth, CA). DNA was sheared to a mean size of 200 bp using a model S220 sonicator (Covaris Inc, Woburn, MA) and prepared into libraries using ThruPLEX DNA-Seq Kits (Takara Bio USA, San Jose, CA). Libraries were pooled in equimolar fashion and sequenced at a read length of 2×150 bp on an Illumina NovaSeq 6000 instrument using a S4 300-cycle kit (Illumina, San Diego, CA) to a mean coverage of 18× for normal samples (range, 15×-21×) and 26× for tumor samples (range, 14×-43×). Sequencing data processing and variant calling were performed as described.[16]

Plasma Multiplex TARDIS

TARDIS assay primer pools were designed to target 36 somatic variants per patient, as identified by tumor/normal WGS. After oligonucleotide production, pools were functionally tested using TARDIS assays on sheared control human cell line DNA. Primer pools that passed this quality control were used in the TARDIS assay to probe 2.6-21.1 ng of cell-free DNA isolated from 3.7 to 5.3 mL of matched patient plasma using the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) as described.[16]

TARDIS libraries were sequenced at a read length of 2×100 bp on an Illumina NovaSeq 6000 instrument using S1 200-cycle kits. Paired-end sequencing reads were analyzed using the TARDIS data analysis pipeline and aligned to human genome hg19 using BWA-MEM as described.[16]

Statistical Methods

Patients were treated with an ICI until disease progression or discontinuation because of adverse events, death, or subject/investigator decision. Response to therapy was assessed per clinician's evaluation of computerized tomography of the chest, abdomen, and pelvis. Clinicians were blinded to ctDNA results at the time of radiographic assessments.

Comparison of ctDNA values between groups was made using the Kruskal-Wallis test. Patient characteristics were compared between PR and CR patients with the Fisher exact test or Wilcoxon rank-sum test for discrete and continuous variables, respectively. The significance threshold for type I error was set at 0.05. SASV9.4 software program was used to perform statistical analyses.

RESULTS

Patient Characteristics

Of 23 patients enrolled, 10 patients did not have sufficient tissue available for WGS and one patient was unable to be evaluated via the TARDIS platform because of suboptimal primer development, leaving 12 patients for ctDNA analysis. Of these 12 patients, 8 (67%) were male and 4 (33%) were female, with a median age of 63 years (range, 54-73). Median lines of therapy received was 1.5 (range, 1-4), median duration of therapy was 24.4 months (range, 7.7-61.3), and median follow-up was 93 days (range, 80-133); no patients were lost to follow-up. Most patients had clear cell histology (92%) and were International Metastatic RCC Database Consortium (IMDC) intermediate risk (92%). All patients had previously received a nephrectomy before initiation of systemic therapy. Treatment was equally distributed between nivolumab plus ipilimumab (50%) and nivolumab monotherapy (50%). Nine patients (75%) achieved a PR, and three patients (25%) achieved a CR. Upon subsequent scans, six patients who previously achieved a PR experienced disease progression. A summary of patient baseline characteristics is presented in Table 1.

TABLE 1. Patient Demographics and Baseline Characteristics

Characteristic	Partial Response (n = 9)	Complete Response (n = 3)	*P*
Age, years, median (range)	66 (56-73)	58 (54-63)	.1
Sex, No. (%)			.2
Male	7 (78)	1 (33)	
Female	2 (22)	2 (67)	
Histology, No. (%)			1.0
Clear cell	6 (67)	2 (67)	
Variant histology	3 (33)	1 (33)	
IMDC risk category, No. (%)			1.0
Favorable	1 (11)	—	
Intermediate	8 (89)	3 (100)	
Line of therapy, median (range)	2 (1-4)	1 (1-2)	.4
Line of therapy, No. (%)			1.0
First-line	4 (44)	2 (67)	
Second-line	3 (33)	1 (33)	
Further lines	2 (22)	—	
Treatment, No. (%)			1.0
Nivolumab	5 (56)	1 (33)	
Nivolumab + ipilimumab	4 (44)	2 (67)	
Duration of response, months, median (range)	22.7 (7.7-61.3)	11.2 (7.7-25.9)	

Abbreviation: IMDC, International Metastatic RCC Database Consortium.

Tumor Genomic Profiling

Using WGS to identify candidate genes for our bespoke assay, an average of 30 patient-specific mutations (range, 19-35) were identified for the quantification of VAFs. Multiplexed sequencing achieved a mean target coverage of 103,342 reads. We also characterized tumor mutational profiles via whole exome sequencing (WES) as part of routine care. The most frequently altered genes in our series were *VHL* (67%), *SMARCA4* (25%), *BAP1* (17%), *PBRM1* (17%), *SETD2* (17%), and *TSC1* (17%). A summary of mutation frequency, mutational signatures, and frequently mutated genes is shown in Figure 1.

ctDNA Detection via Ultrasensitive Multiplex Polymerase Chain Reaction–Based Next-Generation Sequencing

Across our 12 patients, median cell-free DNA yield was 5.1 ng/mL of plasma (range, 2.6-21.1 ng/mL). ctDNA was detected in all but one patient at baseline (median, 0.101%, range, 0%-11.315%). The difference in ctDNA concentration when compared by sex (male *v* female), age (<65 *v* ≥65 years), type of therapy (nivolumab *v* nivolumab plus ipilimumab), or histologic subtype (clear cell *v* variant) was not statistically significant. Clinical characteristics are depicted in Figure 2.

Of the 12 patients in the series, those with a CR had a significantly lower ctDNA concentration than patients with a PR (median, 0.007% [IQR, 0.0%-0.028%] *v* 0.181% [IQR, 0.077%-0.420%], respectively [*P* = .014]). Additionally, six patients demonstrated radiographic progression subsequent to ctDNA assessment. Patients who progressed on subsequent scans had significant higher baseline ctDNA than those who maintained their response (median, 0.362% [IQR, 0.181%-2.71%] *v* 0.033% [IQR, 0.007%-0.077%], respectively [*P* = .026]). VAF analysis is summarized in Figure 3.

DISCUSSION

To our knowledge, this pilot study represents the first ctDNA assay to effectively discriminate between PRs and CRs to immunotherapy in mRCC. Our findings suggest that TARDIS may be an effective bespoke platform for monitoring molecular residual disease and relapse in mRCC.

Development of a reliable ctDNA assay for mRCC has historically been a formidable challenge. One prevailing theory is that the low detection rates in RCC—which range

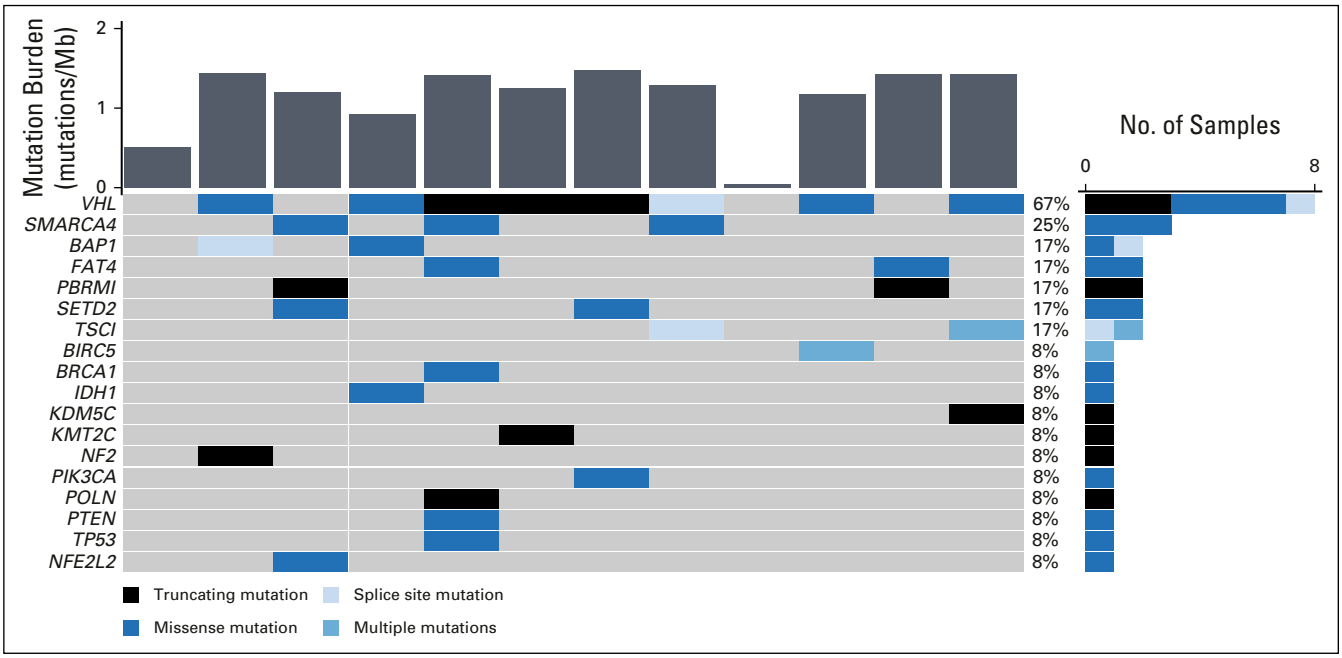

FIG 1. Summary of tumor molecular profiles. Tumor mutational burden (top) along with mutations in frequently mutated genes (bottom) and their relative frequencies (right) for all patients.

from 30% to 40% in older studies using tumor-agnostic assays—are due to low levels of ctDNA shed into the plasma.[18-20] However, in the largest assessment of ctDNA in mRCC to date, our group detected genomic alterations in 71.8% of patients,[21] commensurate to rates seen in other tumor types.[22] Using TARDIS, a targeted tumor-informed approach, yielded higher assay sensitivity and specificity.

Unfortunately, previous efforts using such a tumor-informed approach in mRCC have encountered comparably low yields to their tumor-agnostic counterparts. Using a commercially available bespoke assay guided by tumor WES, Correa et al detected ctDNA in 14 of 34 (41%) patients with RCC of varying stage.[23] After definitive surgical resection, 16 of 33 patients deemed ctDNA-negative by the assay subsequently relapsed, corresponding to a negative predictive value of 52%. Similarly, Jang et al[24] prospectively applied the aforementioned bespoke assay to five patients with mRCC starting on ICI therapy. These authors reported a

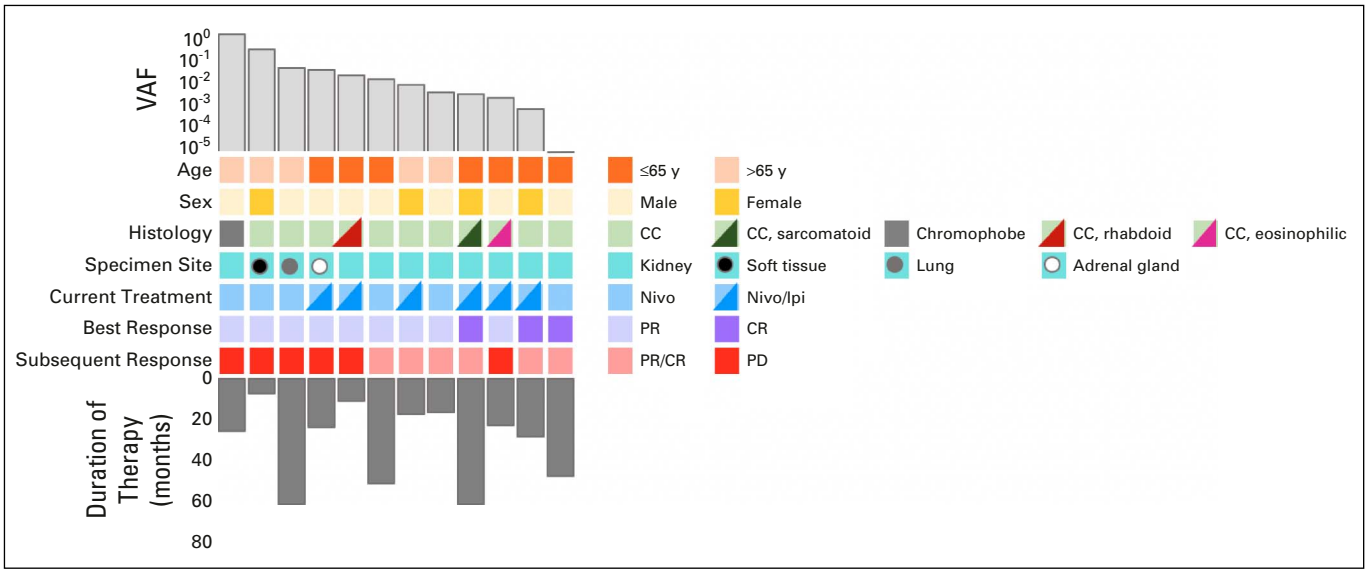

FIG 2. Clinicopathologic and molecular characteristics. Relevant clinical features and VAFs for all patients. CC, clear cell; CR, complete response; Nivo, nivolumab; Nivo/Ipi, nivolumab plus ipilimumab; PD, progressive disease; PR, partial response; VAFs, variant allele fractions.

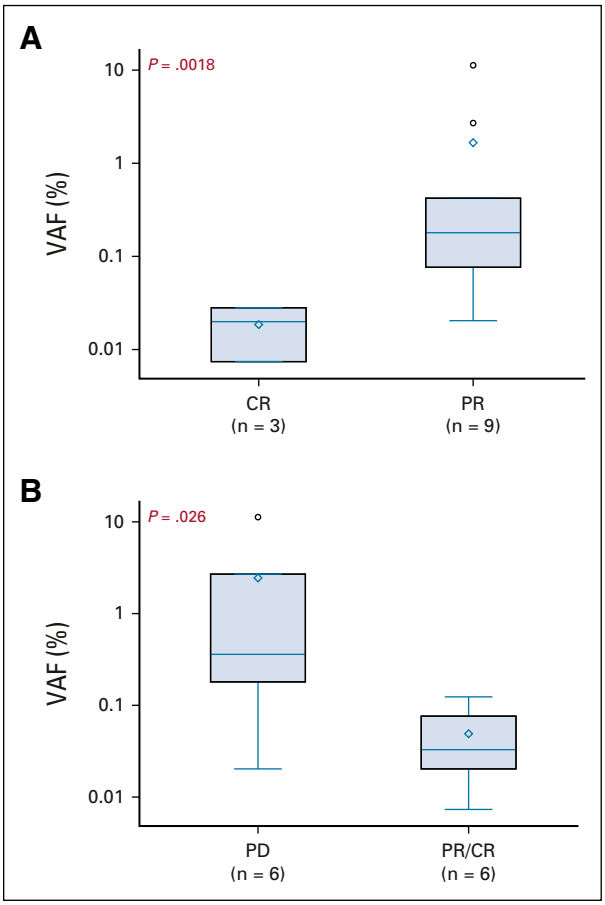

FIG 3. Association of ctDNA with clinical outcomes. (A) Discrimination of PR and CR using TARDIS. (B) Discrimination of sustained radiographic response versus subsequent progression. CR, complete response; ctDNA, circulating tumor DNA; PD, progressive disease; PR, partial response; TARDIS, targeted digital sequencing.

concordance rate between ctDNA response and radiographic response of only 60%. One potential explanation for the higher apparent predictive value of TARDIS may lie in our application of WGS rather than WES. By encompassing entire tumor genomes for the development of our bespoke ctDNA panels, we were also able to target noncoding aberrations that would otherwise be overlooked via WES, enabling a greater number of mutations analyzed in plasma DNA.

Our assay builds upon a growing body of evidence that suggests larger quantities of genomic targets may enhance the depth of ctDNA detection. One such commercially available 16-gene assay reached a limit of detection of 0.034% VAF in patients receiving pembrolizumab.[25] Another assay, which uses up to 48 tumor-specific variants, reported ctDNA detection at levels as low as 26 parts per million (equivalent to 0.0026% VAF) in patients with postsurgical head and neck squamous cell carcinoma.[26] In between lies TARDIS, which in our study incorporated an average of 30 patient-specific mutations and achieved a detection limit of 0.007% VAF. Taken together, these data support the premise that increasing the quantity of patient-specific mutations augments the effective depth of sequencing per sample, improving the sensitivity and quantitative precision for ctDNA analysis.

As previously noted, a modest proportion of patients on ICIs will mount a CR to ICI-based combination therapy, and an even larger subset may have durable PRs. Presumably, patients with a higher ctDNA concentration after initiation of systemic therapy would be candidates for continuation of treatment, and those with a lower ctDNA concentration might be spared further treatment. Although some have proposed using a durable radiographic response as a decision point for treatment discontinuation,[27,28] there are obvious limitations with this approach—our study, for instance, highlights the detectability of molecular residual disease even in those patients with a radiographic CR. Additionally, it is challenging to ascertain the presence of active disease in certain sites of metastasis—one prominent example is bone metastases, where a sclerotic reaction can be challenging to differentiate from disease progression.

ctDNA also has potential application in earlier settings. In RCC, the phase III KEYNOTE-564 supports the role of adjuvant pembrolizumab in patients with high-risk localized RCC.[29] Presumably, those patients with higher ctDNA burden would have derived greater benefit from therapy. This principle has borne out in muscle-invasive bladder cancer, where a distinct ultrasensitive test has been shown to predict clinical benefit from adjuvant therapy with the ICI atezolizumab using samples collected from a randomized, phase III study (ImVigor010).[30] These data have led to the inception of ImVigor011 and TOMBOLA, both of which are biomarker-based randomized trials in muscle-invasive bladder cancer that will allocate treatment with adjuvant atezolizumab on the basis of the presence or absence of ctDNA (NCT04660344 and NCT04138628, respectively).

One of the limitations of this study was its nonrandomized observational design using an institutional database. In an effort to mitigate selection bias, all patients who met the inclusion criteria were included in this study. Another limitation was our high rate (43%) of insufficient tissue for WGS processing, which was primarily because these biopsies were consumed for unrelated research endeavors that predated the study herein. In addition, our sample size was small, lacked survival data, and incorporated one sample at varying time points on therapy; therefore, these data should be viewed as hypothesis-generating. However, we hope to remedy these limitations with a larger prospective validation study of patients with metastatic disease starting on ICI that is currently ongoing at our institution. Blood will be collected at baseline and at consistent time points during therapy. If meaningful distinctions in ctDNA are identified among patients with differing clinical response (eg, CR *v* PR, as in the current study), we will initiate studies assessing discontinuation of treatment in patients with low ctDNA levels after ICI therapy.

In conclusion, to our knowledge, the data presented herein are the first published report of TARDIS, an ultrasensitive ctDNA assay, for disease surveillance in mRCC. By employing a bespoke approach that uses WGS to identify an average of 30 patient-specific mutations, TARDIS was able to effectively differentiate patients who achieved a PR from those who achieved a CR with immunotherapy. These significant differences seen in ctDNA, if validated in larger series, imply that the assay may play a role in facilitating treatment discontinuation.

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DATA SHARING STATEMENT

Genomic data from tissue and plasma specimens will be deposited at the Translational Genomics Research Institute (TGen) and will be available upon request. The authors defer depositing the participant genomic data in national and international public repositories because of institutional policies and the absence of statements in patient consent forms that would have allowed controlled access distribution and genomic data availability. Deidentified individual participant genomic libraries and clinical data that underlie the results reported in this article are available for transfer on a specific secure server housed at the TGen. Interested investigators can obtain and certify the data transfer agreement (DTA) and submit requests to the principal investigator, S.K.H. Proposals will be vetted by the TGen Data Access Committee. Investigators and institutions who consent to the terms of the DTA form, including but not limited to the use of these data for the purpose of a specific project and only for research purposes, and to protect the confidentiality of the data and limit the possibility of identification of participants in any way whatsoever for the duration of the agreement, will be granted access. TGen will then facilitate the transfer of the requested deidentified data. This mechanism is expected to be via an Aspera High-Speed File Transfer Server but TGen reserves the right to change the specific transfer method at any time, provided appropriate levels of access authorization and control can be maintained.

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APPENDIX 1

TABLE A1. Patient Treatment History

Patient	Age (years)	Sex	Subtype	First-Line Treatment	Second-Line Treatment	Third-Line Treatment	Fourth-Line Treatment	Tumor Burden (mm)	Best Response	Next Response
1	68	Male	Chromophobe	Sunitinib	Nivolumab			133	PR	PD
2	69	Female	CC	Nivolumab	Pazopanib			118	PR	PD
3	56	Male	CC, eosinophilic	Nivo/Ipi				85	PR	PD
4	73	Male	CC	Sunitinib	Sorafenib	Sonepcizumab	Nivolumab	74	PR	PD
5	63	Male	CC, rhabdoid	Nivo/Ipi				59	PR	PD
6	62	Male	CC	Nivo/Ipi				46	PR	PD
7	59	Male	CC	Sunintib	Nivolumab			44	PR	PR
8	73	Male	CC	Cabozantinib	Len/Ev	Nivolumab		43	PR	PR
9	66	Female	CC	Nivo/Ipi				39	PR	PR
10	54	Female	CC, sarcomatoid	Nivo/Ipi				0	CR	CR
11	63	Female	CC	Nivo/Ipi				0	CR	CR
12	58	Male	CC	Sunitinib	Nivolumab			0	CR	CR

Abbreviations: CC, clear cell; CR, complete response; Len/Ev, lenvatinib/everolimus; Nivo/Ipi, nivolumab/ipilimumab; PD, progressive disease; PR, partial response.