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Detection of von Hippel-Lindau gene mutation in circulating cell-free DNA for clear cell renal cell carcinoma

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Abstract

The therapeutic landscape of metastatic clear cell renal cell carcinoma (ccRCC) has rapidly expanded, and there is an urgent need to develop noninvasive biomarkers that can select an optimal therapy or evaluate the response in real time. To evaluate the clinical utility of circulating tumor DNA (ctDNA) analysis in ccRCC, we established a highly sensitive assay to detect mutations in von Hippel-Lindau gene (*VHL*) using a combination of digital PCR and multiplex PCR–based targeted sequencing. The unique assay could detect *VHL* mutations with a variant allele frequency (VAF) <1.0%. Further, we profiled the mutation status of *VHL* in 76 cell-free DNA (cfDNA) and 50 tumor tissues from 56 patients with ccRCC using the assay. Thirteen *VHL* mutations were identified in cfDNA from 12 (21.4%) patients with a median VAF of 0.78% (range, 0.13%-4.20%). Of the 28 patients with *VHL* mutations in matched tumor tissues, eight (28.6%) also had *VHL* mutation in cfDNA with a median VAF of 0.47% (range, 0.13%-2.88%). In serial ctDNA analysis from one patient, we confirmed that the VAF of *VHL* mutation changed consistent with tumor size by radiographic imaging during systemic treatment. In conclusion, *VHL* mutation in cfDNA was detected only in a small number of patients even using the highly sensitive assay; nevertheless, we showed the potential of ctDNA analysis as a novel biomarker in ccRCC.

KEYWORDS

biomarkers, cell-free DNA, clear cell renal cell carcinoma, next-generation sequencing, VHL

1 | INTRODUCTION

Renal cell carcinoma (RCC) is the seventh most common cancer in the developed countries and accounts for 2.2% of all cancer incidences.[1] One third of the patients with RCC show metastases at diagnosis, and one quarter relapse with distant metastases after curative nephrectomy.[2] Since the 2000s, multiple treatment options for metastatic RCC (mRCC) have come into practice including vascular endothelial growth factor (VEGF) inhibitors, mTOR inhibitors, and immune checkpoint inhibitors (ICIs).[2] Combination regimens that simultaneously target VEGF and immune checkpoints have also been approved in 2019.[3,4] As the treatment landscape of mRCC grows in complexity and an increasing number of treatment options become available, defining strategies based on tumor biology to select the

right therapy for the right individual becomes crucial. Additionally, modalities other than radiographic imaging, such as computed tomography (CT) and MRI, are required to monitor response to therapy because these imaging systems are costly and time-consuming; moreover, in the case of CT scanning, patients are exposed to substantial radiation doses. Therefore, biomarkers that can select the optimal therapy or evaluate the response in real time are urgently required.

Advances in sequencing techniques have clarified the genomic and transcriptomic landscape of RCC using primary and metastatic tissues.[5-8] A majority of the information on RCC genomics has been obtained from studies of clear cell RCC (ccRCC), which is the major histological subtype of RCC (75%-80%). The commonly altered genes in ccRCC include von Hippel-Lindau gene (*VHL*) and chromatin remodeling genes, such as Polybromo 1 gene (*PBRM1*), SET domain–containing 2 gene (*SETD2*), and BRCA1-associated protein 1 gene (*BAP1*), which are located close to *VHL* on the short arm of chromosome 3. Some of these alterations are reported to have prognostic and predictive roles.[9-12] However, it is impractical to routinely obtain fresh metastatic tissues for genotyping because of biopsy-related complications, costs, and technical issues that may limit access to biopsy sites. Sequencing of archival primary tissue may also be less relevant to metastatic tumors treated with prior systemic therapies.

Liquid biopsies comprising circulating tumor cells and circulating tumor DNA (ctDNA) have emerged as alternatives to tissue biopsy.[13] CtDNA is a subset of cell-free DNA (cfDNA) that is released from tumor cells into the bloodstream. CtDNA analysis addresses tumor heterogeneity by potentially capturing genomic information that is representative of more than one or different metastatic sites and at multiple time points throughout the treatment. However, the major limitation of ctDNA analysis is their variable abundance even in patients with metastatic disease. Moreover, unlike other genitourinary malignancies, only few reports are available for ctDNA analysis in RCC. Some reports have also shown that the detection rate of ctDNA is only 30%.[14,15] Therefore, it still remains uncertain whether the ctDNA abundance in RCC patients is sufficient for developing a broad genotyping platform.

To discriminate ctDNA from total cfDNA, genomic footprints, such as tumor-specific mutations, need to be detected with very high sensitivity. *VHL* is one of the major driver genes most frequently mutated at an early phase in ccRCC.[5,8,16] Therefore, we hypothesized that *VHL* mutation analysis would be a first step in the development of a ctDNA assay to assess the presence of ctDNA in ccRCC. In the present study, we first aimed to establish a highly sensitive assay to detect *VHL* mutations using a combination of digital PCR (dPCR) and multiplex PCR–based targeted sequencing. Second, we profiled the *VHL* status in plasma cfDNA in patients with ccRCC and investigated clinical categories correlated with *VHL* mutations in cfDNA and evaluated their clinical utility.

2 | MATERIALS AND METHODS

2.1 | Patient cohort

Fifty-six patients with ccRCC and 31 healthy men without a history of malignancy were recruited at the Kyoto University Hospital, Japan between September 2015 and March 2019. Both metastatic and nonmetastatic patients with histologically confirmed ccRCC were eligible for this study. The metastatic cohort included patients with at least one distant metastatic lesion. Patients with or without prior nephrectomy were eligible, and the cohort included those who were responding to systemic therapy and those who have progressed on a systemic therapy. The nonmetastatic cohort were mostly locally advanced clinical tumor stage (T3 or T4); however, two patients with clinical T2N0M0 were also included. All human experiments were approved by the ethical committee of the Kyoto University Hospital (approval number: G1083). Written informed consent was obtained from all the patients. All human experiments were performed in accordance with the Japanese Ethical Guidelines for Human Genome/Gene Analysis Research and Ethical Guidelines for Medical and Health Research involving Human Subjects. Details of blood collection, processing, and DNA extraction from blood and tissue are described in Appendix S1.

2.2 | VHL mutation analysis using targeted sequencing

VHL mutation analysis was performed using dPCR and multiplex PCR–based targeted sequencing. Basically, cfDNA were initially evaluated by targeted sequencing. However, since the intrinsic error rate of next-generation sequencing (NGS) is approximately 1%, we validated mutation candidates with variant allele frequency (VAF) <1% by subsequent dPCR to exclude false-positive calls. For targeted sequencing, a total of eight primer sets spanning the *VHL* exon region were designed and grouped into three sets for multiplex PCR. First, multiplex PCR was performed using 2 ng of DNA and Q5 Hot Start High-Fidelity DNA polymerase (NEB) per reaction. Successful amplification was confirmed by agarose gel electrophoresis, and the PCR products were purified by AMPure XP beads (Beckman Coulter). Next, overhang adapters specifically designed for Illumina sequencing were attached to the purified first-PCR amplicons using primers used for the first PCR with an overhang adapter sequence (second PCR). After purification, the concentration of each set of second PCR amplicons was measured using Qubit 3.0 Fluorometer, and the amplicons were pooled for each sample. Finally, a limited cycle amplification was performed to attach sample-specific barcodes to the second PCR amplicons using Q5 Hot Start High-Fidelity DNA polymerase and the Nextera XT Index Kit (Illumina; third PCR). Agarose gel electrophoresis, purification, and concentration measurements were performed, and PCR products from all the 96 samples were pooled. Library quality and quantity were evaluated using the Bioanalyzer

TABLE 1 The sensitivity of *VHL* mutation analysis using targeted sequencing and dPCR

	RCC4 (C > G, S65W)		A498 (del TGAC, G144fs*)	
Mixture ratio (%)/ estimated VAF (%)	VAF by targeted sequencing (%)	VAF by dPCR (%)	VAF by targeted sequencing (%)	VAF by dPCR (%)
100/100	99.8, 99.9	99.9, 100	98.7, 99.8	97.8, 99.4
66/50	47.9, 49.1	53.8, 55.1	38.8, 41.6	37.2, 39.2
20/11.1	11.6, 11.9	11.1, 13.1	7.0, 9.1	6.0, 6.3
10/5.3	6.1, 6.5	6.2, 6.4	3.1, 3.5	3.1, 3.2
2.0/1.0	0.9, 1.6	0.9, 1.2	0.6, 0.9	0.5, 0.6
1.0/0.5	0.7, 0.7	0.3, 0.4	not detected	0.2, 0.2
0/0	Not detected	Not detected	Not detected	Not detected

Note: DNA from two RCC cell lines with VHL mutation (RCC4 [C > G, S65W] and A498 [del TGAC, G144fs*]) were used. Estimated VAF were calculated from the hypothesis that VHL mutation was accompanied by loss of heterozygosity in the cell lines. The experiments were performed in duplicate, and each result is shown.

Abbreviations: dPCR, digital PCR; RCC, renal cell carcinoma; VAF, variant allele frequency.

2100 and quantitative PCR (qPCR) according to the Illumina qPCR Quantification Protocol Guide, and the libraries were normalized. Sequencing was performed on Illumina MiSeq system according to the manufacturer's instructions. The sequencing run included serially diluted DNA extracted from two RCC cell lines as positive controls, randomly selected white blood cell (WBC) DNA from 16 patients with ccRCC, and cfDNA from five healthy men as negative controls.

Paired-end reads were aligned against the human reference genome (GRCh37) using Burrows-Wheeler Aligner, and the resulting files were converted into the pileup format by SAMtools.[17,18] For candidate single-nucleotide variants (SNVs) and short indel detection, the following criteria were applied: (i) VAF ≥0.5% after removing base calls with base quality or mapping quality <20; (ii) minimum five variant-supporting reads; (iii) minimum 1000 read depth; and (iv) if a variant was not consistent between paired-end reads, both reads were discarded. After variant candidate calling, the candidates were filtered based on the sequence data of the WBC DNA. The mean frequency of candidate variants in the WBC DNA was considered as the error rate. Each VAF in cfDNA was compared with the error rate using one-sided binomial test, and the variant was discarded when $P > .05$. Additionally, we examined whether there was strand bias at the candidate positions. We compared the genotypes inferred from the positive strand with those from the negative strand using the chi-square test; the candidate was discarded if $P < .05$. We further filtered the variant candidates for more stringent mutation calling by setting the cutoff of VAF to 1.0% for cfDNA and 5.0% for tumor tissue DNA. For the candidates in cfDNA with VAF between 0.5% and 1.0%, the variants validated with dPCR were considered to be true. Additionally, the variants detected in matched tumor tissue DNA were considered to be true even if the VAF was <1.0% in cfDNA.

2.3 | VHL mutation analysis using dPCR

VHL mutation analysis by dPCR was performed using the QuantStudio 3D Digital PCR system (Thermo Fisher Scientific). PCR reaction was prepared with 7.5 μL of QuantStudio3D Digital PCR master mix (Thermo Fisher Scientific), 0.75 μL of custom-made Taqman SNP genotyping assay (Thermo Fisher Scientific) that represents FAM and VIC signal for mutation and wild, respectively, and DNA (approximately 5 ng for cfDNA and 10 ng for DNA from cell line, tumor tissue, and WBC) in a total volume of 15 μL. The PCR reaction was loaded onto QuantStudio3D Digital PCR Chip (Thermo Fisher Scientific) and amplified on ProFlex 2x Flat PCR System (Thermo Fisher Scientific). After PCR amplification, chips were read on QuantStudio3D Digital PCR Instrument (Thermo Fisher Scientific), and a secondary analysis was performed using QuantStudio3D Analysis Suite Cloud software (Thermo Fisher Scientific). CfDNA samples were considered positive for target mutations if they contained at least one plot in the FAM signal region of scatter plots. To minimize the risk of false-positive calls in cfDNA analysis, we applied more than double input DNA for WBC analysis compared with cfDNA analysis, and confirmed that no mutant plots were detected in WBC DNA from healthy men in the development of the dPCR assay (Table 1). Further, no mutant plots were observed in all the experiments using matched WBC DNA in the validation analysis (Figure S2). We have applied the same method in our previous study.[19] VAF by dPCR was defined as the proportion of copies of mutation relative to the sum of copies of mutation and wild.

2.4 | RCC cell lines

A498 cell lines were purchased from the American Type Culture Collection. RCC4 cell line was kindly provided by Dr William G Kaelin Jr (Dana-Farber Cancer Institute, Boston, MA).[20] DNA from each cell line was extracted using a procedure similar to that used for tumor tissue DNA. Cell line DNA was sequentially diluted with WBC DNA from a healthy male and used as a positive control to assess the sensitivity of the established assay. The VAF of the *VHL* mutation in each diluted DNA has been pre-estimated based on the hypothesis that *VHL* mutation in the cell lines is accompanied by a loss of

heterozygosity. Therefore, the mixture ratio of cell line DNA to normal DNA (R) and the estimated VAF are related as: VAF = (R × 1)/[(1−R) × 2 + R × 1]. All experiments using cell lines were performed in duplicate.

2.5 | Statistical analysis

The cfDNA yield between patients with ccRCC and healthy men or patients with castration-resistant prostate cancer (CRPC) was compared using the Mann-Whitney rank test. The correlation between cfDNA yield and each clinical category was analyzed using the Mann-Whitney rank test. The VAF of *VHL* mutations in cfDNA from patients with ccRCC was compared with that of androgen receptor gene (*AR*) mutations in cfDNA from patients with CRPC using the Mann-Whitney rank test. Clinical categories associated with the presence of *VHL* mutation in cfDNA were analyzed using univariate analysis (Fisher's exact test for categorical variables and logistic regression for continuous variables). Overall survival (OS) after the first blood collection was estimated using the Kaplan-Meier method, and differences in OS between patients with and without *VHL* mutation in cfDNA were compared using the log-rank test. For calculating cfDNA yield and VAF of *AR* mutations in patients with CRPC, we referenced our previous study on ctDNA in CRPC.[19] Differences were considered to be statistically significant when $P < .05$. Data analyses and statistical tests were performed in Python V3.7, using pandas, numpy, scipy, and lifelines survival analysis.

3 | RESULTS

3.1 | Analyses of VHL mutations using targeted sequencing and dPCR

VHL mutation analysis was first performed using two RCC cell lines to ensure that two mutation types (point mutation and insertion/deletion) could be detected. *VHL* S65W was detected until RCC4 DNA was diluted to 1.0% (estimated VAF 0.5%) (Table 1). *VHL* G144fs* was also detected using targeted sequencing and dPCR when A498 DNA was diluted to 2.0% (estimated VAF 1.0%) and 1.0% (estimated VAF 0.5%), respectively (Table 1). No other suspected false-positive mutation candidates were detected in the diluted DNA from the cell lines. *VHL* in cfDNA from five healthy men was also sequenced using the same assay. Three mutation candidates with VAF <1.0% were detected using targeted sequencing; however, two of them were undetectable using dPCR, indicating false positives (Table S1 and Figure S1). Therefore, we set the threshold of VAF to 1.0% for *VHL* mutation analysis using targeted sequencing of human cfDNA samples. Mutation candidates with VAF <1.0% were regarded as true mutations if they were validated using dPCR or detected in matched tumor tissue DNA.

3.2 | Patient characteristics and cfDNA yield in patients with ccRCC

Fifty-six patients with ccRCC were recruited in this study, and 76 blood samples and 50 tumor tissue samples were collected (Figure 1A). Sixteen patients had two or more blood samples. Tumor tissue samples, collected from 39 patients, comprised 45 primary and five metastatic sites (adrenal, bone, subcutaneous tissue, and opposite kidney). Patient characteristics at baseline are shown in Table 2, Figure 1B, and Table S2. Twenty-eight patients (50%) underwent nephrectomy before the first blood collection. Twenty-two (39.3%) patients were receiving systemic therapy such as VEGF inhibitors, and four of them were confirmed with therapy resistance by radiographic imaging at the first blood collection. All 14 patients without metastases had unresected primary kidney tumors at baseline, and 12 of them had clinical stage T3 or T4 disease. Twenty-four patients with metastases who were not on systemic therapy at baseline or were switched to other systemic therapy due to disease progression after first blood collection were evaluated by the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk group.[21,22] Eighteen of the 24 patients were stratified into intermediate- or poor-risk groups.

The median cfDNA yield at baseline was 11.6 ng/mL of plasma (range, 3.4-128 ng/mL). This was significantly higher than that in healthy men (11.6 vs. 5.52 ng/mL plasma, $P < .001$, Mann-Whitney rank test) and was similar to that in patients with CRPC studied previously (11.6 vs. 11.4 ng/mL plasma, $P = .58$, Mann-Whitney rank test) (Figure 2A).[19] We investigated the correlation between cfDNA yield and the clinical categories (Figure 2B). Patients in the IMDC poor-risk group had significantly higher cfDNA yield than those in the favorable-risk group (25.5 vs. 5.92 ng/mL plasma, $P = .011$, Mann-Whitney rank test) and intermediate-risk group (25.5 vs. 11.6 ng/mL plasma, $P = .017$, Mann-Whitney rank test). Patients who were receiving systemic therapy also tended to have a lower cfDNA yield. There was no obvious association between cfDNA yields and the presence of distant metastases.

3.3 | Evaluation of VHL status in cfDNA

VHL targeted sequencing was performed on 74 of the 76 cfDNA samples (Figure 1A). The remaining two cfDNA were sequentially collected from one patient, kyt056, to track the change in VAF of *VHL* mutation using dPCR. The median coverage of each amplicon was 23174X. Sixteen *VHL* mutation candidates were detected in 13 patients by targeted sequencing, 12 of which were VAF <1.0% (Table 3). Nine of the 12 were validated by dPCR and/or in matched tumor tissue (Table 3 and Figure S2). The remaining three candidates that were not validated by dPCR or in matched tumor tissue were regarded as false positives. Therefore, 13 *VHL* mutations were identified in 12 of 56 (21.4%) patients by the

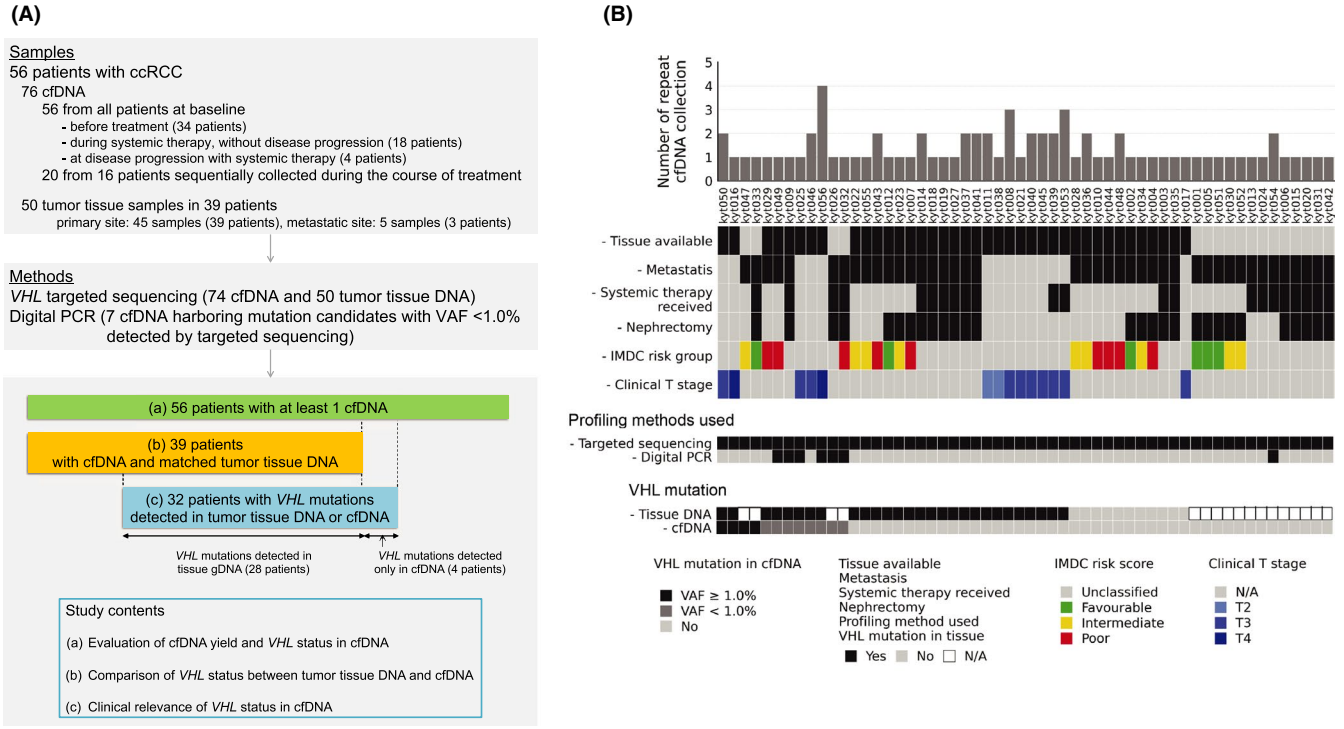

FIGURE 1 A, Study flow chart. B, Overview of patient clinical characteristics at baseline, profiling methods used, and *VHL* status in tissue DNA and cfDNA. Horizontal bar lengths in (A) are proportional to sample size (annotated). Patients in (B) are sorted by *VHL* status in tissue DNA and cfDNA. ccRCC, clear cell renal cell carcinoma; cfDNA, cell-free DNA; IMDC, International Metastatic Renal Cell Carcinoma Database Consortium

TABLE 2 Patients' characteristics at baseline

Characteristics		All patients (n = 56)	Patients with matched tumor tissue (n = 39)
Age, years	Mean (range)	70.3 (43-85)	69.3 (43-85)
Gender, male/female	No. (%)	42 (75.0)/14 (25.0)	30 (76.9)/9 (23.1)
Nephrectomy before first blood collection	No. (%)	28 (50)	15 (38.5)
Ongoing systemic therapy	No. (%)	22 (39.3)	11 (28.2)
Distant metastases	No. (%)	42 (75.0)	25 (64.1)
Extraregional lymph node		8 (14.3)	4 (10.3)
Bone		18 (32.1)	12 (30.8)
Lung		27 (48.2)	15 (38.5)
Liver		7 (12.5)	1 (2.6)
Adrenal gland		4 (7.14)	2 (5.1)
Pancreas		4 (7.14)	0 (0)
Other		9 (16.1)	3 (7.7)
IMDC risk group[a]	No. (%)		
Favorable		6 (25.0)	2 (12.5)
Intermediate		9 (37.5)	6 (37.5)
Poor		9 (37.5)	8 (50)

Abbreviation: IMDC, International Metastatic Renal Cell Carcinoma Database Consortium.

[a]IMDC risk group was evaluated in the patients with metastases who were not on systemic therapy at baseline or were switched to other systemic therapy due to disease progression after first blood collection (n = 24).

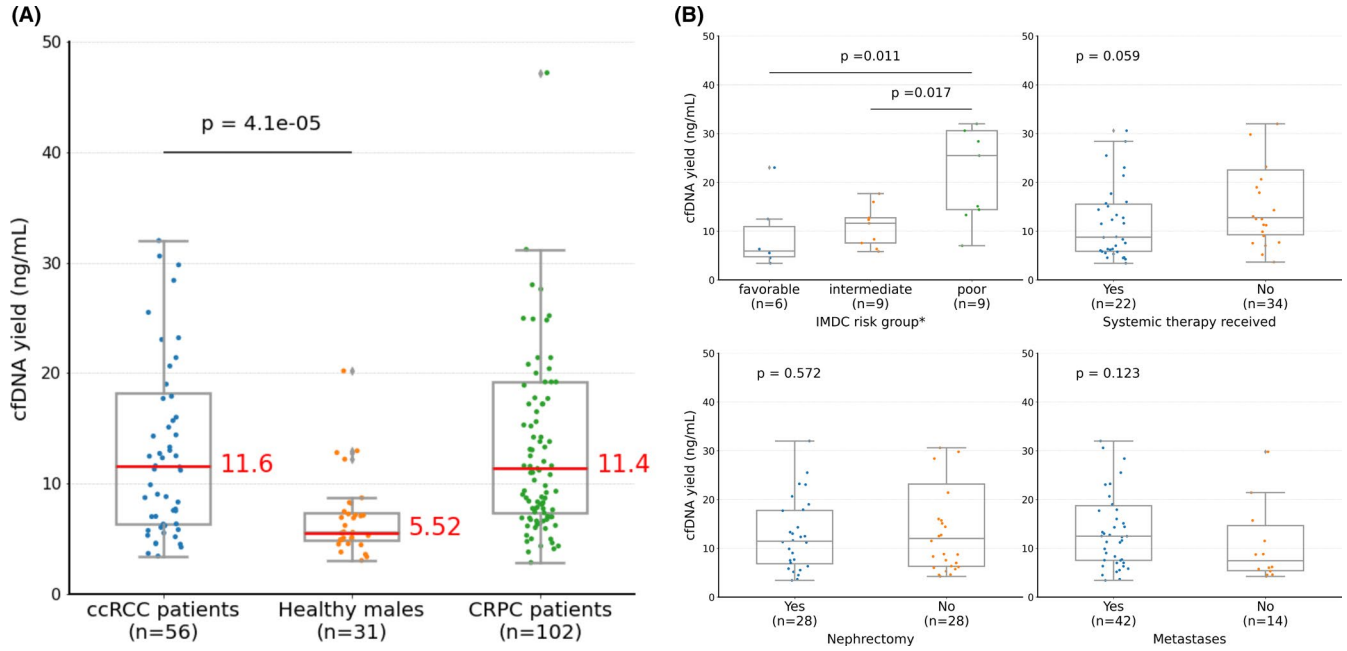

FIGURE 2 Summary of cfDNA yields. A, Median cfDNA yield from patients with ccRCC (n = 56), healthy men (n = 31), and patients with CRPC (n = 102). B, Correlation between cfDNA yields and clinical categories. *IMDC risk group was evaluated in the patients with metastases who were not on systemic therapy at baseline or were switched to other systemic therapy due to disease progression after first blood collection (n = 24). ccRCC, clear cell renal cell carcinoma; cfDNA, cell-free DNA; CRPC, castration-resistant prostate cancer; IMDC, International Metastatic Renal Cell Carcinoma Database Consortium

combination of targeted sequencing and dPCR, of which six were truncating mutations (Figure 3A and Table 3). Similar to previous sequencing results using tumor tissues,[5,6] *VHL* mutations in cfDNA were evenly distributed in the present study, and there were no hot-spot mutations within *VHL*. The median VAF of *VHL* mutations by targeted sequencing was 0.78% (range, 0.13%-4.20%), and the frequency was significantly lower than that of *AR* mutations in patients with CRPC (0.78% vs. 1.43%, $P = .0076$, Mann-Whitney rank test) (Figure 3B).[19] Patients kyt009 and kyt025 had H115Q in cfDNA confirmed by both targeted sequencing and dPCR, but not in matched tumor tissue DNA, possibly reflecting tumor heterogeneity.

3.4 | Exploration of clinical categories associated with the presence of VHL mutation in cfDNA

We next focused on patients with *VHL* mutation in tumor tissue DNA to determine which clinical categories are associated with detection of *VHL* mutation in cfDNA. Of the 39 patients whose matched tumor tissues were sequenced, 28 (71.8%) had 31 *VHL* mutations, 17 of which were truncating mutations (Figure 3A and Table S3). The VAF of *VHL* mutation in patients kyt049 and kyt050 was <5.0%, but these mutations were regarded as true because the mutation in kyt049 was validated using dPCR and that in kyt050 was shared in primary tumor tissues sequentially obtained with biopsy and nephrectomy (Figure S2 and Table S3). Similar to the distribution of *VHL* mutations detected in cfDNA, the *VHL* mutations in tumor tissue DNA were also evenly distributed. In three patients, metastatic tumor tissues shared the same *VHL* mutation detected in the corresponding primary tumor tissue. Of the 28 patients with *VHL* mutations in tumor tissues, eight (28.6%) had *VHL* mutations in cfDNA with a median VAF of 0.47% (range, 0.13%-2.88%) (Figure 3C). One (kyt009) of the eight patients had a *VHL* mutation in cfDNA, which was different from that in matched tumor tissue. Importantly, the remaining 11 patients without *VHL* mutations in tumor tissue exhibited no *VHL* mutation in cfDNA. Next, clinical categories associated with the presence of *VHL* mutations in cfDNA were analyzed in the 28 patients by univariate analysis (Table S4). No significant categories linked to the detection of *VHL* mutation in cfDNA were observed because of the small sample size, but the absence of primary disease and ongoing systemic therapy at the time of cfDNA collection could be inversely associated with detection of *VHL* mutation in cfDNA.

3.5 | Clinical relevance of VHL mutation in cfDNA

We examined the clinical relevance of *VHL* mutations in cfDNA in 32 patients with *VHL* mutations in tumor tissue DNA or cfDNA (Figure 1A). The remaining 24 patients with no mutations in either tumor tissue or cfDNA were excluded from this analysis. Thirteen of the 32 patients had no distant metastases, and 11 of them underwent nephrectomy after the first blood collection (three patients were treated with axitinib as a neoadjuvant therapy). Additional cfDNA samples were collected after nephrectomy in two patients (kyt046

TABLE 3 Summary of VHL mutations and false positives in cfDNA from patients with ccRCC

Patient ID	Sample ID	Start position	End position	Reference	Alteration	Total reads	Variant reads	Amino acid change	VAF by targeted sequencing (%)	VAF by dPCR (%)	Detection of same mutation in matched tissue
VHL mutation											
kyt033	cfDNA1300	10183725	10183725	C	T	25488	1070	S65L	4.2	n/a	n/a
kyt016	cfDNA1226	10188201	10188201	A	T	13596	391	H115L	2.88	n/a	Yes
kyt047	cfDNA1323	10183857	10183857	T	A	35314	742	I109N	2.1	n/a	n/a
kyt050	cfDNA1347	10183867	10183867	C	-	33799	598	R113fs	1.77	n/a	Yes
kyt025	cfDNA1052	10188263	10188263	T	-	19322	160	F136fs	0.16	0.07	Yes
	cfDNA1052	10188202	10188202	C	A	34833	54	H115Q	0.83	0.61	No
kyt026	cfDNA1023	10188207	10188207	G	C	22606	188	W117S	0.83	0.74	n/a
kyt032	cfDNA1069	10191652	10191652	A	G	29856	234	- (3'-UTR)	0.78	0.79	n/a
kyt009	cfDNA1059	10188202	10188202	C	A	24698	147	H115Q	0.6	0.98	No
kyt049	cfDNA1326	10183872	10183872	G	A	20431	96	X114_splice	0.47	1.1	Yes
kyt046	cfDNA1306	10191470	10191470	G	C	41100	76	X155_splice	0.18	n/a	Yes
kyt029	cfDNA1130	10191493	10191493	C	A	18574	31	C162*	0.17	n/a	Yes
kyt056	cfDNA1374	10188260	10188261	TT	-	42994	56	L135fs	0.13	0.15	Yes
False positives											
kyt026	cfDNA1023	10183682	10183682	G	T	31488	278		0.88	Not detected	n/a
kyt054	cfDNA1396	10183627	10183627	G	T	24070	136		0.57	Not detected	n/a
	cfDNA1396	10191479	10191479	C	G	41021	238		0.58	Not detected	n/a

Abbreviations: ccRCC, clear cell renal cell carcinoma; cfDNA, cell-free DNA; dPCR, digital PCR; VAF, variant allele frequency.

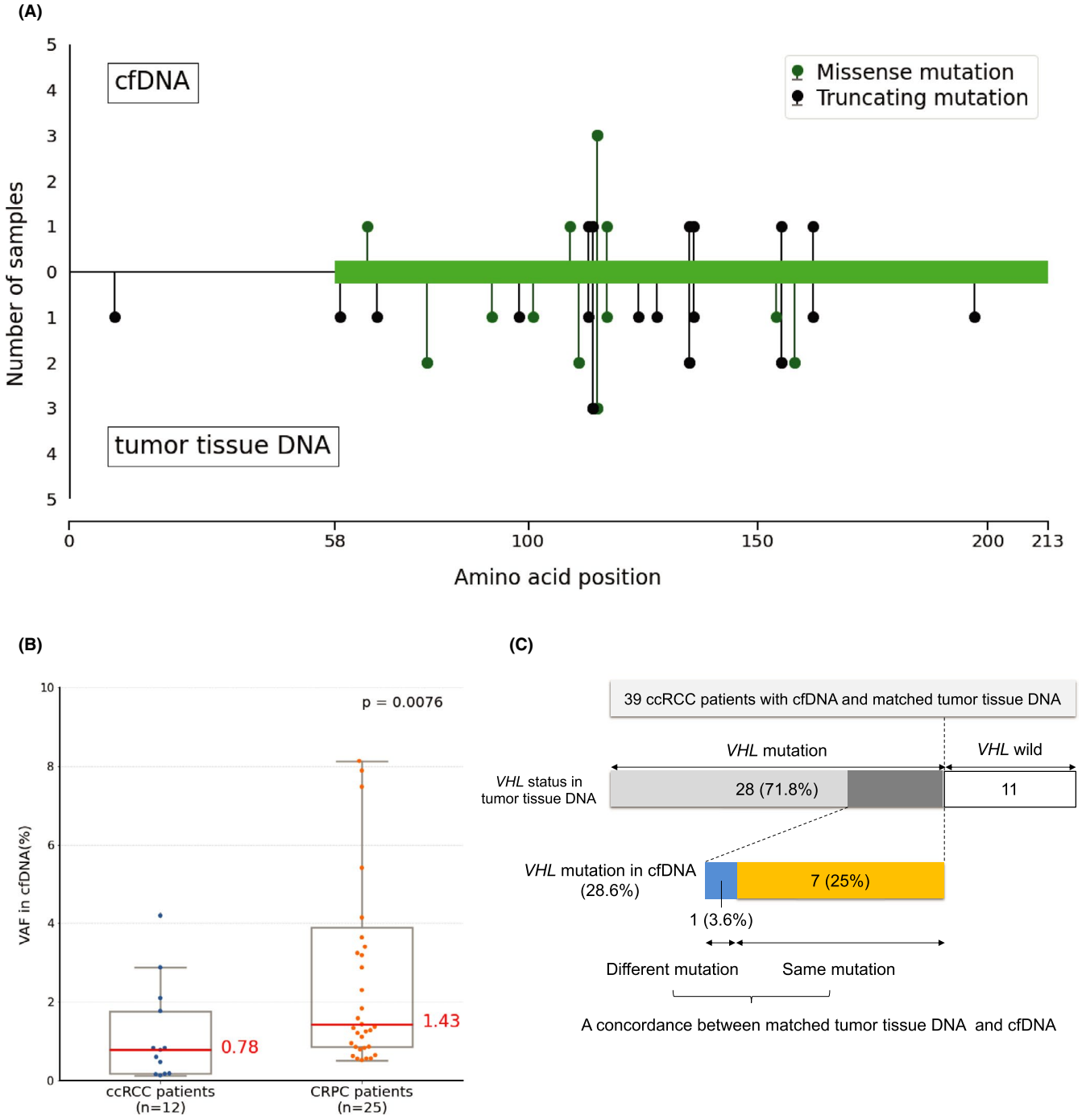

FIGURE 3 Identification of *VHL* mutations in cfDNA and tumor tissue DNA. A, Lollipop plots showing *VHL* mutations mapped to protein domains (upper: cfDNA, lower: tumor tissue DNA). B, Box plots for VAF of *VHL* and *AR* mutations by targeted sequencing in cfDNA from patients with ccRCC and CRPC, respectively. C, Flowchart of selection process for identifying patients with *VHL* mutation common in cfDNA and matched tumor tissue from 39 patients with ccRCC. One patient has a *VHL* mutation in cfDNA that is different from mutation detected in matched tumor tissue. Horizontal bar lengths are proportional to sample size (annotated). *AR*, androgen receptor gene; ccRCC, clear cell renal cell carcinoma; cfDNA, cell-free DNA; CRPC, castration-resistant prostate cancer; VAF, variant allele frequency

and kyt050) with *VHL* mutations in both tumor tissue DNA and preoperative cfDNA (Table 3). These patients had advanced clinical and pathological tumor stage tumors, but the *VHL* mutation became undetectable after nephrectomy. Moreover, in patient kyt050, the tumor invading the inferior vena cava has not recurred for 2 years after nephrectomy. Patient kyt056, with clinical T4N2M0 tumor harboring *VHL* mutation in baseline cfDNA, was treated with VEGF inhibitors, pazopanib and axitinib, and the cfDNA samples were sequentially collected to track the VAF of the *VHL* mutation by dPCR (Figure 4). Pazopanib was switched to axitinib due to treatment-related grade 3 liver dysfunction. While the primary tumor size decreased with pazopanib and axitinib, the VAF in cfDNA temporarily

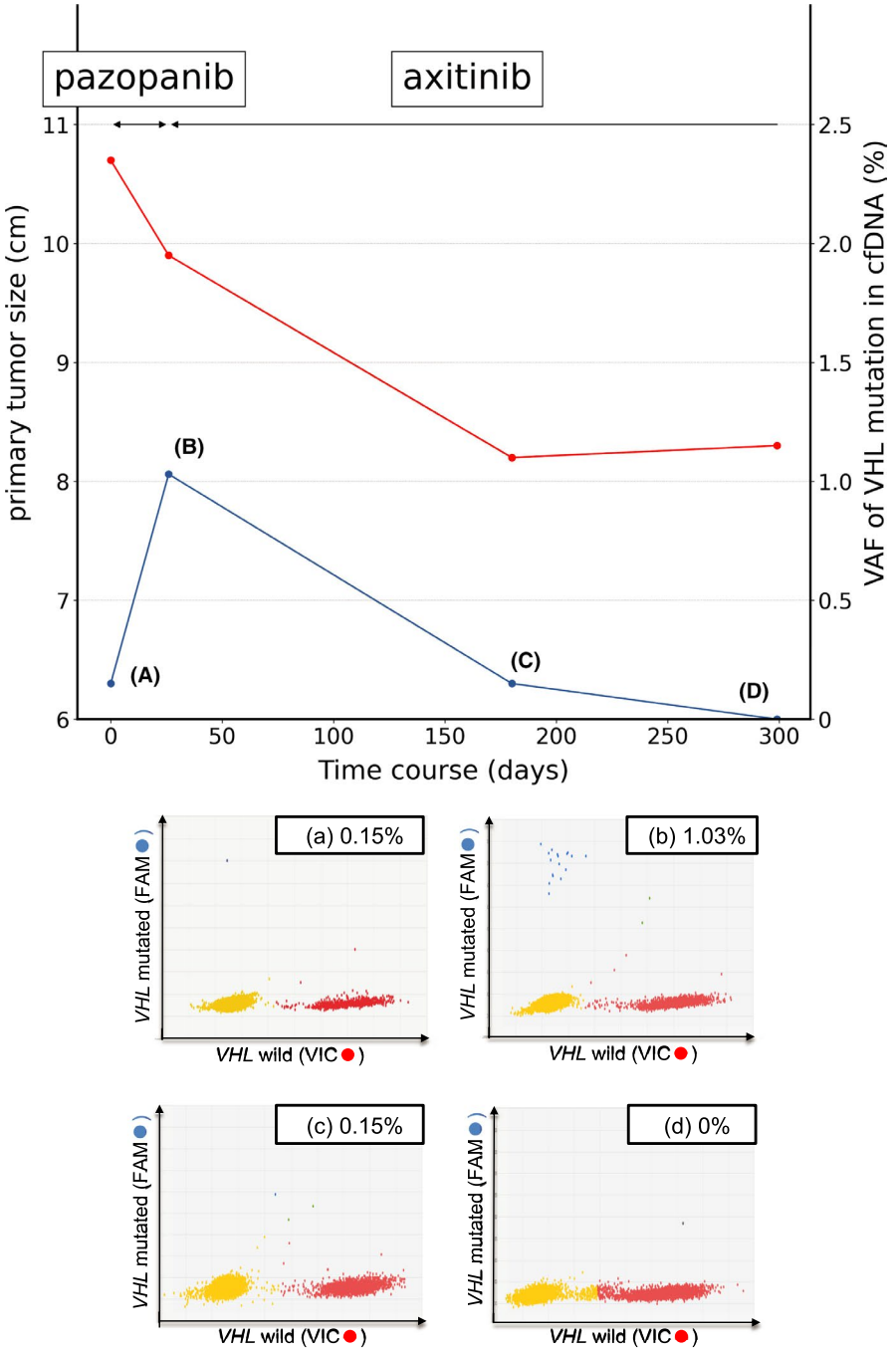

FIGURE 4 A representative case with ccRCC showing the feasibility of sequential analysis of *VHL* mutation in cfDNA. The red and blue lines indicate changes in the primary tumor size and VAF of *VHL* mutation in cfDNA by digital PCR, respectively. Scatter plots show *VHL* mutation analysis in serially collected cfDNA by digital PCR. The blue dots show positive droplets for the *VHL* mutation; red dots show positive droplets for the *VHL* wild type; and yellow dots show empty droplets. The value at the top of each panel represents VAF. ccRCC, clear cell renal cell carcinoma; cfDNA, cell-free DNA; VAF, variant allele frequency

increased after initiation of pazopanib and then decreased serially to an undetectable range at the fourth cfDNA collection.

As for the remaining 19 patients with distant metastases, they received systemic therapies such as VEGF inhibitors, ICIs, surgery, or radiotherapy for primary or metastatic sites. Eleven of the 19 patients were treated with new therapy after baseline blood collection, and five of them had *VHL* mutation in cfDNA. Despite a small cohort and short follow-up (median 14.0 months), those five patients tended to show a shorter OS than the remaining six patients with *VHL* mutation only in tissue DNA ($P = .10$, log-rank test) (Figure S3).

4 | DISCUSSION

CtDNA analysis has multiple potential roles, including detection of treatment-resistant clones, selection of targeted therapy, and monitoring response to therapy in several cancer types. Many patients with other metastatic genitourinary cancer types have significant ctDNA to profile genomic status in cfDNA.[23-25] Prospective biomarker-driven trials are currently underway for metastatic CRPC to select optimized treatments based on the results of ctDNA analysis (NCT03385655, NCT03903835, and NCT04015622).[26-28] However, the clinical utility of ctDNA analysis remains understudied

in RCC, and RCC is reported to release the least amount of cfDNA among all the extracranial tumors.[29] The largest study comprising 220 patients with mRCC, including 89 patients with ccRCC, revealed that genomic alterations in cfDNA were detected in 79% of patients undergoing Clinical Laboratory Improvement Amendments–certified Guardant360 ctDNA test (Guardant Health).[30] However, the mutations showed a median VAF of 0.2%, of which some may be derived from unrelated somatic expansion, such as benign clonal blood lineage, as the commercial test fails to analyze matched WBC DNA as control. Indeed, a recent study analyzing ctDNA observed somatic mutations in clonal hematopoiesis of indeterminate potential (CHIP)-related genes in one third of the patients with mRCC.[15] In contrast, other studies showed that only 30% of ccRCC patients had evidence of ctDNA by targeted cfDNA sequencing that captures the coding regions of cancer-related genes.[14,15] However, detection of ctDNA in these studies could have been limited by a low sequence depth and higher cutoff (1.0%) for calling somatic mutations.

To capture the full landscape of mutations in cfDNA from patients with ccRCC, we first developed an assay to detect mutations with lower allele frequency by deep sequence depth focusing on *VHL*, as it is the most commonly mutated gene in sporadic ccRCC and its mutation occurs at an early stage.[8,16] NGS can be used to comprehensively detect genomic alterations, such as mutations, insertions, and deletions, without requiring any previous knowledge of molecular alterations. However, detection of mutations with low allele frequency is challenging because standard NGS technology has an intrinsic error rate of 1%, making it difficult to distinguish between true and false positives below this limit. In the present study, targeted sequencing detected false-positive base changes with VAF <1.0% in cfDNA from three healthy men. However, two of them could not be validated by dPCR, indicating false positives. dPCR is usually more sensitive and specific than NGS and can detect rare mutations with a prevalence as low as 0.1%. In the present and our previous study, dPCR could detect mutations with low VAF in highly diluted cell line DNA.[19] Therefore, we applied dPCR to complement targeted sequencing for low-frequency variants. Additionally, parallel assessment of WBC DNA in this setting by dPCR helped us exclude any variants derived from CHIP. The unique combination of targeted sequencing and dPCR in this study could detect *VHL* mutations with VAF <1.0% in diluted DNA from RCC cell lines and cfDNA from patients with ccRCC.

The frequency of *VHL* mutation in tumor tissue DNA in the present study was comparable to that in other studies.[5-7] However, although we developed a very sensitive cfDNA assay, *VHL* mutation was detected in cfDNA from only 28.6% of patients harboring *VHL* mutations in tumor tissue DNA. Additionally, even when *VHL* mutation was detected in cfDNA, the median VAF was significantly lower than that of *AR* mutations in cfDNA detected by a similar assay in patients with CRPC[19]; this was contrary to our expectations, as we had hypothesized that *VHL* mutations would be detected more frequently in ccRCC characterized by hypervascularization and often accompanied by a large area of necrosis, allowing continuous shedding of cfDNA. Several explanations can be given for the low detection rate and VAF of ctDNA in the present study. First, the present cohort included some patients treated with systemic therapy at baseline. A previous study demonstrated that systemic therapy rapidly reduced ctDNA levels in patients with metastatic prostate cancer.[24] In the present study, *VHL* mutation in cfDNA was detected in only one patient treated with systemic therapy without disease progression at baseline, suggesting that systemic therapy inhibited ctDNA release in these patients. Second, 75% of the patients in our cohort had distant metastases at blood collection; however, the overall tumor burden may have been insufficient to detect *VHL* mutations in cfDNA. A significant correlation was observed between ctDNA abundance and metrics of overall tumor burden in other cancers.[23] The present study also revealed that *VHL* mutation in cfDNA tended to be detected more frequently in patients who had not undergone nephrectomy at baseline, supporting the possible correlation between ctDNA detection rate and tumor burden. Third, it is possible that the amount of ctDNA present in the bloodstream varies with different genetic regions. Previous studies on cfDNA in mRCC have also failed to detect *VHL* mutations in tissues at the expected frequency (50%-60%); however, they showed a significantly higher frequency of mutations in certain genes such as *TP53* and *PIK3CA* that are rarely detected in primary ccRCC specimens.[30] Although the exact mechanism is unknown, ctDNA at *VHL* regions may not be abundant in the blood stream either by decreased shedding or early degradation. Future studies are required to directly compare the frequency of *VHL* mutations and other mutations relevant to mRCC using a highly sensitive assay.

This study showed the potential clinical utility of cfDNA analysis for ccRCC despite the low detection rate of *VHL* mutations in cfDNA. First, we showed that VAF of *VHL* mutation in cfDNA changed consistent with tumor size by radiographic imaging during systemic treatment in a patient with ccRCC. As in a previous report, the transient increase in VAF after initiation of pazopanib may reflect tumor lysis syndrome.[31] With a widespread use of immunotherapy, there is an emergence of pseudoprogression related to radiographic evaluation. This could lead to inappropriate treatment decisions, and hence, sequential tracking of mutated gene copies by dPCR may provide additional information for assessing tumor burden.[32] Second, we showed that cfDNA yield was significantly higher in the poor-risk patients in the IMDC risk category. Although a previous study has reported higher cfDNA yield in metastatic disease than in localized disease, the present study showed no association.[33] The result of the present study may have been influenced by the fact that 20 of the 42 patients with metastatic sites were on systemic therapy at the time of baseline cfDNA collection. Third, even though limited by a small number of patients and heterogeneous patient population, our data suggested that patients with *VHL* mutations in cfDNA might have worse OS than those without such mutations. Similar trends have been observed in two studies in which cfDNA from patients with RCC were sequenced.[14,15] Although future confirmation in a larger cohort is necessary, these findings suggest that cfDNA yield and detection of *VHL* mutation in cfDNA may be a prognostic marker in ccRCC, and that serial tracking of *VHL* mutation could be used as a biomarker to complement radiographic assessment in these aggressive diseases.

Importantly, drugs targeting hypoxia-inducible factor 2α (HIF2α), which accumulates in tumor cells with VHL defect and plays a role as the main driver of ccRCC, have been developed and a phase III trial is currently ongoing (NCT04195750).[20,34,35] Multiple preclinical and translational studies have also started to elucidate potential biomarkers of efficacy of HIF2α inhibition. Therefore, *VHL* mutation as well as that of *EPAS1*/*HIF2A* in cfDNA might be a predictive biomarker for the targeting therapy or could be used as a marker to track treatment response.

This study had several notable limitations. First, the sample size was small, and cfDNA was collected from a heterogeneous patient cohort. Thus, the results of ongoing larger prospective studies will further demonstrate the clinical utility of cfDNA analysis in mRCC (NCT03469713 and NCT03414827). Second, although this study focused on *VHL* mutations, there are other genomic alterations that may be useful as predictive and prognostic biomarkers. Several studies have shown that VEGF inhibitors and everolimus significantly prolong OS or progression-free survival in patients with *PBRM1* mutations detected in tumor tissues.[10-12] NGS-based analysis using molecular barcodes allows comprehensive detection of mutations with VAF below 1.0% and could be used in the future to study genomic alterations associated with ccRCC pathogenesis.[36] Additionally, tumor mutational burden (TMB) could predict response to ICIs in other cancers.[37-39] CfDNA sequencing is a practical tool to determine patients with hypermutated metastatic prostate cancer and could potentially be used to infer TMB in RCC.[40] Furthermore, recent studies have also demonstrated the feasibility of methylation analysis in cfDNA in RCC.[41,42] Taken together, future biomarker development efforts using cfDNA in RCC must expand to encompass aspects of the epigenome and beyond.

In conclusion, we analyzed the *VHL* status of cfDNA in patients with ccRCC. Only 30% of the patients harboring *VHL* mutation in tumor tissue DNA had *VHL* mutation detected in cfDNA, even with a VAF cutoff <1.0%. However, the present study also showed the potential of cfDNA analysis in ccRCC as a useful biomarker in some clinical settings.

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DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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