Characteristics of circulating-tumor DNA in non-metastatic clear cell renal cell carcinoma

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Characteristics of circulating-tumor DNA in non-metastatic clear cell renal cell carcinoma

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ABSTRACT

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Background: Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma. Circulating-tumor DNA (ctDNA) has emerged as a biomarker which complements or acts as an alternative to renal mass biopsy. However, the characteristics of ctDNA have not been elucidated in non-metastatic ccRCC, especially with small tumors.

Methods: Plasma was preoperatively collected from 120 patients who underwent surgical resection for suspected kidney cancer. Samples of ccRCC were sequenced using next-generation sequencing and sequenced data were analyzed using the Pi-Seq algorithm (Dxome, Sungnam, Korea). The characteristics of ctDNA were compared between non-metastatic ccRCC and metastatic ccRCC. Non-metastatic ccRCC was stratified according to pathological T (pT) stage into pT1a and pT1b-3a, and associations with ctDNA were further investigated. The detection rate, variant allele frequency, and proportion of genes with ctDNA were evaluated. ctDNA was investigated in association with several clinicopathological features of ccRCC. The positive concordance of somatic variants between plasma and matched tissue was evaluated.

Results: Of the 120 patients included in this study, 90 were diagnosed with ccRCC, 20 were diagnosed with non-ccRCC, and 10 were diagnosed with benign

tumors. Among the 90 patients with ccRCC, 15 were excluded based on their medical history; and of the remaining 75 patients, non-metastatic ccRCC was confirmed in 67 patients. Most non-metastatic ccRCC cases (79.1%) were classified as pT1a ccRCC. Detection rates of ctDNA were 26.9% and 75.0% in non-metastatic and metastatic ccRCC, respectively. The detection rate of non-metastatic ccRCC showed a tendency to increase as the tumor size increased. The detection rate of ctDNA in pT1a ccRCC was 22.6%. Median variant allele frequencies of ctDNA were 0.351% and 1.168% in non-metastatic and metastatic ccRCC, respectively. The proportion of genes with ctDNA in non-metastatic ccRCC was different from that of metastatic ccRCC. *VHL*, *PBRM1*, *SETD2,* and *BAP1* were frequently detected in metastatic ccRCC while *NF1*, *TP53,* and *KDM6A* were frequently detected in non-metastatic ccRCC. ctDNA detection in non-metastatic ccRCC was associated with tumor sizes and patient age, but not with tumor grade. ctDNA was frequently detected when lymphovascular invasion, fat tissue invasion, or venous tumor thrombus were concurrently observed, but the associations were not statistically significant. Positive concordance between ctDNA and matched tissue was poor in non-metastatic ccRCC.

Conclusions: The characteristics of ctDNA in non-metastatic ccRCC were explored, with particular attention on small-sized pT1a ccRCC. Low detection rate, low variant allele frequency, and different proportion of genes with ctDNA were demonstrated in non-metastatic ccRCC compared with metastatic ccRCC. ctDNA was associated with tumor size and patient age in non-metastatic ccRCC. However, the relationship between ctDNA and tumor grade was not clear. Possible variants of clonal hematopoiesis were not filtered, which was a limitation of this study. A prospective study is required to demonstrate the clinical significance of ctDNA in non-metastatic ccRCC. Improvement of the sensitivity of ctDNA analysis and filtration of clonal hematopoiesis may advance the clinical utility of ctDNA.

Key words: circulating-tumor DNA, clear cell renal cell carcinoma, non-metastatic cancer, small renal mass

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I. INTRODUCTION

Renal cell carcinoma (RCC), a type of cancer that originates from the epithelial cells of the kidney, accounts for more than 90% of all kidney cancers.[1] More than ten subtypes of RCC have been reported, the majority of which are categorized as clear cell RCC (ccRCC), papillary RCC, or chromophobe RCC.[2] ccRCC, the most common subtype of RCC, is responsible for the majority of deaths attributed to kidney cancer.[3] Kidney cancer accounts for more than 2% of all cancers worldwide, and approximately 400,000 patients are newly diagnosed with kidney cancer each year.[4]

The 5-year survival rate of localized RCC is 50-90%, which is significantly higher than the 5-year survival rate of metastatic ccRCC, which is less than 13%. Nephrectomy is mainly performed for non-metastatic ccRCC. However, despite surgery, 30% of patients eventually develop metastases. Due to the poor outcome of metastatic RCC, risk stratification at diagnosis is necessary for proper management.[5] Moreover, as sonography and computed tomography (CT) are widely used, incidental findings of small renal masses (≤ 4 cm in maximal

diameter) have increased, and now account for more than half of newly diagnosed cases of RCC.[6] Because approximately 20% of small renal masses are benign, and 60% are malignant but tend to be indolent with low metastatic potential, active surveillance and focal ablation have been introduced as strategies for the management of small renal masses in older patients with considerable comorbities.[7,8] Notably, the remaining 20% of small renal masses are malignant with unfavorable characteristics.[9,10]

The histological subtype and grade of RCC are associated with disease progression and metastatic potential. ccRCC has the fastest growth rate and patients diagnosed with this subtype are more likely to develop metastasis than those with other RCC subtypes.[9,11] Various studies examining the RCC grade report that RCC with a high nuclear grade shows aggressive features and is related to poor prognosis.[12]

In this context, biopsy of suspicious renal masses should be considered prior to the development of a suitable treatment plan. However, several limitations should be addressed.[10,13] First, up to 14% of renal mass biopsies are non-diagnostic. Second, a renal mass biopsy may not fully characterize the entire renal mass due to intra-tumoral heterogeneity. Third, although renal mass biopsy in surgical specimens show acceptable concordance with histopathology results, the concordance of grade is less reliable. Last, safety and tumor seeding issues still remain associated renal mass biopsies, especially when biopsies are performed for renal masses with cystic changes.[9,13-15] Therefore, several studies have been conducted to find alternative or complementary methods for renal mass biopsy and ultimately discover new biomarkers for RCC diagnosis and risk stratification.[16]

Circulating-tumor DNA (ctDNA) is found in the bloodstream and refers to DNA derived from tumor cells. As ctDNA contains information on genetic modifications in cancer cells, it is a potential biomarker for several cancers, and has various applications for the diagnosis, monitoring, treatment, and

prognostication of these cancers.[17] Moreover, this non-invasive method can capture the whole genetic heterogeneity and burden of cancer.[18]

Many studies have been conducted to investigate the clinical application of ctDNA analysis in RCC, the majority of which have focused on ccRCC. To date, discussion has primarily focused on the ctDNA mutational profile of ccRCC and its concordance with matched tissue samples.[19,20] The prognostic value of ctDNA in ccRCC has also been investigated.[19,21] However, because most ctDNA studies investigated metastatic ccRCC, very little information is available about the characteristics of ctDNA in non-metastatic ccRCC.[19-21] Furthermore, to the best of our knowledge, a ctDNA analysis for small renal masses with a large number of patients has never been conducted. In the present study, we demonstrate the characteristics of ctDNA in non-metastatic ccRCC. Small ccRCC masses with a diameter less than 4 cm were further investigated and compared with non-metastatic ccRCC masses with a diameter greater than 4 cm, and metastatic ccRCC.

II. MATERIALS AND METHODS

1. Samples and study design

Ethics approval was obtained from the Ethics Committee of Yonsei University College of Medicine in Seoul, Korea (approval no: IRB No: 4-2019-1039). A total of 120 patients with suspicious renal masses or metastatic lesions scheduled to be surgically removed were enrolled in this study. Peripheral blood was collected immediately before surgical resection. Blood samples were aliquoted into ethylenediaminetetraacetic acid-containing tubes, centrifuged at 1600×g for 10 min at 4°C, and then transferred to fresh tubes. Samples were further centrifuged at 4000×g for 10 min at 4 °C. Plasma samples were stored at -80 °C until ctDNA analysis.

After medical record review, patients with non-ccRCC were excluded from this study, and patients with ccRCC or benign tumor were included. Patients with

RCC with other subtypes (such as papillary, chromophobe, or clear cell papillary type) or non-RCC malignancies (such as mixed epithelial, stromal tumor, or Wilms tumor) were allocated to the non-ccRCC group. Patients with oncocytoma, angiomyolipoma, or benign cyst were allocated to the benign tumor group. Patients with metastatic ccRCC were included to compare ctDNA characteristics with those of non-metastatic ccRCC. Patients with a prior history of cancer or chronic kidney disease were excluded to minimize factors that could influence ctDNA detection.[22,23]

Clinicopathological data including age, sex, past medical history, and pathological information about surgically removed mass, as well as imaging studies were obtained via chart review. Pathological data included tumor size, histological type, tumor grade, tumor extent, lymphovascular invasion (LVI), fat tissue invasion (FTI), and venous tumor thrombus (VTT). Tumor size was measured using the maximum diameter of the tumor. Histological subtype was assessed according to the 2016 edition of the World Health Organization (WHO) histological classification of renal tumors. The tumor grade was determined according to the Furhman grading system or the World Health Organization/International Society of Urological Pathology (WHO/ISUP) grading system. The stage was assessed based on the 8th edition of the American Joint Committee on Cancer Cancer Staging Manual. Imaging studies, including CT, magnetic resonance imaging, and whole-body bone scans, were used to assess tumor size, tumor extent, and regional and distant metastasis. Tumor size and patient age were collected as clinical information for the benign control group.

Tumor tissue was obtained and stored as frozen or formalin-fixed paraffin-embedded (FFPE) samples. Eight available samples, including one benign tumor sample, were selected to compare the concordance of somatic variants between plasma and tissue.

The characteristics of ctDNA were investigated based on the clinicopathological data. Detection rate, median variant allele frequency (VAF),

and proportion of genes with ctDNA were evaluated. Primary tumor size, tumor grade, LVI, FTI, VTT, and patient age were also investigated in association with ctDNA detection. ctDNA from non-metastatic ccRCC samples were compared with ctDNA from metastatic ccRCC samples. Non-metastatic ccRCC was sub-dividend according to pathological T (pT) stage into pT1a and pT1b-3a ccRCC, and the characteristics of ctDNA were compared between groups. Positive concordance of somatic variants between ctDNA and matched tissue was investigated in the eight selected patients. An oncoplot for exploring characteristics of ctDNA and another oncoprint plot for identifying positive concordant somatic variants were generated using the maftool package (http://bioconductor.org/packages/release/bioc/html/maftools.html) and the Complex Heatmaps package (http://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html), respectively, using R 4.0.3 software.[24,25] Figure 1 shows a schematic representation of the study design.

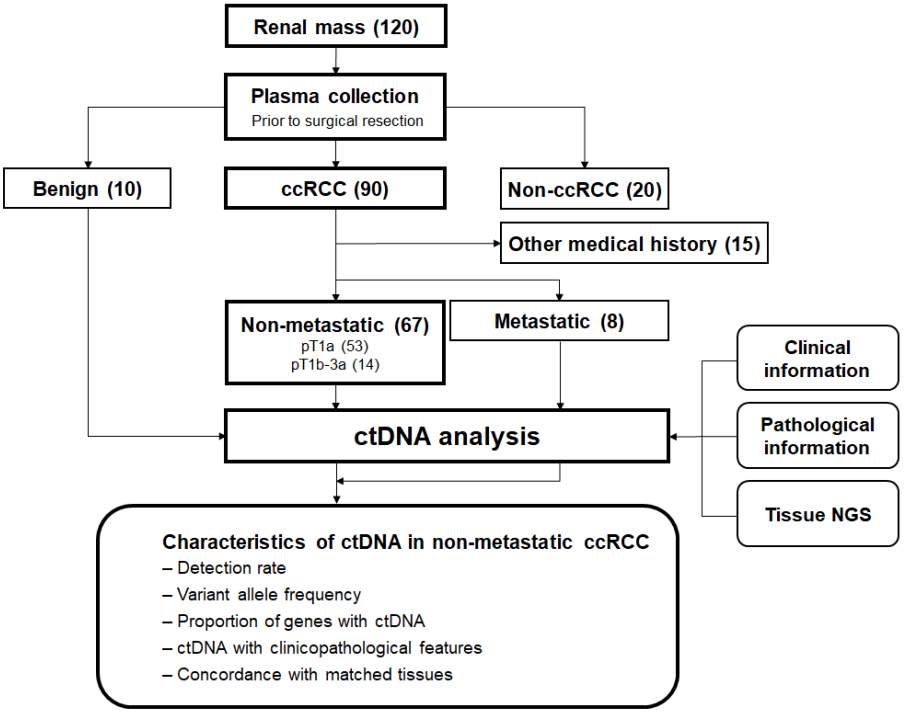

Figure 1. Study design for exploring characteristics of circulating-tumor DNA in non-metastatic clear cell renal cell carcinoma

Plasma was collected from 120 patients who underwent surgical resection for suspicious renal mass or metastatic lesions. Of the 120 patients, 20 had non-ccRCC and 10 had benign tumors. Patients with non-ccRCC were excluded from this study, and patients with benign tumors were included in the benign control group. Of the 90 patients with ccRCC, 15 with past medical history (cancer or chronic kidney disease) were subsequently excluded. Of the remaining 75 patients, 67 had non-metastatic ccRCC (53 with pT1a and 14 with pT1b-3a) and eight had metastatic ccRCC. ctDNA in plasma was detected and annotated using the Pi-Seq algorithm (Dxome, Sungnam, Republic of Korea). By correlating with clinicopathological information, the characteristics of ctDNA in non-metastatic ccRCC were investigated, with particular focus on small-sized ccRCC.

2. Library preparation, target capture, and sequencing for plasma samples

Circulating cell-free DNA (cfDNA) was extracted from 3-4 ml of plasma samples using the Magnetic Serum/Plasma Circulating DNA Kit (Dxome, Sungnam, Korea). The size of cfDNA was measured using the TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA). The cfDNA concentration was measured using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The resulting DNA was ligated using Illumina adapters and indexed using unique dual indices for duplex sequencing (Illumina, San Diego, CA, USA). Sequencing libraries were hybridized with customized probes targeting 16 RCC-related genes which are frequently mutated in RCC, as shown in previous studies (Table. 1).[26-28] Enriched DNA was amplified, and the clusters were generated and sequenced on a NovaSeq 6000 System (Illumina) with 2× 151 bp reads. A mean sequencing depth of 30,000× was targeted. All procedures were performed in accordance with the manufacturer's instructions.

Table 1. Gene list of the target panel for renal cell carcinoma

ARID1A	*BAP1*	*EGFR*	*ELOC*
KDM5C	*KDM6A*	*MET*	*MTOR*
NF1	*NF2*	*PBRM1*	*PIK3CA*
PTEN	*SETD2*	*TP53*	*VHL*

3. Circulating-tumor DNA analysis

The Pi-Seq algorithm (Dxome) was used to call and annotate somatic variants in cfDNA. Pi-Seq is designed to detect true somatic variants using the positional information of aligned uncollapsed reads generated from duplex sequencing. Positional information generated by Pi-Seq is presented as a molecular 'barcode.' When reads arising from the same origin are aligned in the same position, true-positive variants are detected in all aligned reads, whereas false-positive variants are not (Figure 2). Pi-Seq reads were aligned to human genomic reference sequences (GRCh37) using Burrows-Wheeler alignment tool version 0.7.12 (Wellcome Trust Sanger Institute, Cambridge, UK).[29] The HaplotypeCaller and Mutect2 in the genome analysis tool kit (GATK) package version 3.8-0 (Broad Institute of MIT and Harvard, Cambridge, MA, USA) and VarScan2 version 2.4.0 (Washington University, St. Louis, MO, USA) were used to identify SNVs and indels and the results were compared and merged.[30-32] To identify medium to large indels, Pindel 0.2.0 (EMBL Outstation European Bioinformatics Institute, Cambridge, UK) was used.[33] VAF% was calculated as the read depth count of identified variant/total read depth count at the position ×100. All variants were manually inspected using the Integrative Genomic Viewer.[34] ctDNA were selected using the OncoKB^{TM} tumor mutation database which is recognized by the U.S. Food and Drug Administration.[35] Variants of uncertain significance that were not found or rarely found in the normal population and predicted to be pathogenic were also included as ctDNA. The pathogenicity of variants was predicted using multiple computational tools (BayesDel addAF, BayesDel noAF, DANN, DEOGEN2, EIGEN, EIGEN PC, FATHMM, FATHMM-MKL, FATHMM-XF, LIST-S2, LRT, M-CAP, MVP, MutPred, Mutation assessor, MutationTaster, PROVEAN, PrimateAI, SIFT, SIFT4G, and dbscSNV) and only variants predicted to be pathogenic by more than two thirds of the tools were selected. Benign-favor and synonymous variants were excluded.

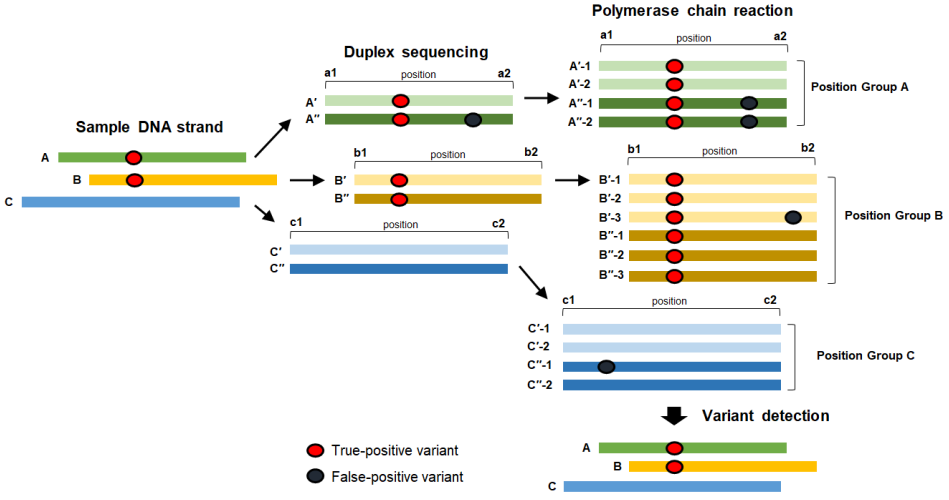

Figure 2. Schematic illustration of Pi-Seq algorithm

Duplex sequencing was performed for sample DNA strands. A polymerase chain reaction with each strand of DNA duplex generates two distinct groups of reads that represent the original DNA strand. Reads originating from the same DNA strand were aligned in the same position group using the start and end positions of each read as a barcode. True-positive variants were detected in all reads in the same position group. False-positive variants were detected in only one or some of the reads.

4. Tissue sequencing

Five frozen tumor tissue samples and three FFPE tumor tissue samples were used for tissue sequencing. DNA was extracted from the frozen tissue samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and sequenced using the Twist Human Core Exome Kit (Twist Bioscience, San Francisco, CA, USA). DNA was extracted from the FFPE tissue samples using the AllPrep FFPE Kit (Qiagen) and sequenced using the TruSight Oncology 500 (Illumina). After hybridization, paired-end DNA sequencing with 2× 151 bp reads was performed for both frozen and FFPE tissue samples using the NovaSeq 6000 System (Illumina). All procedures were performed according to the manufacturer's instructions.

5. Statistical analysis

Fisher's exact test was performed to compare detection rates, the proportion of ctDNA according to tumor grade, LVI, FTI, and VTT, and ctDNA with clonal hematopoiesis (CH)-related gene between groups. The Mann-Whitney test was performed to compare VAF, tumor size, and patient age between groups. All statistical analyses were conducted using the R 4.0.3 software. A *p*-value less than 0.05 was considered statistically significant.

III. RESULTS

1. Patient characteristics

A total of 120 patients who underwent surgical resection for suspicious renal masses or metastatic lesions were initially included in this study. Pathological examination revealed 90 ccRCC diagnoses, 20 non-ccRCC diagnoses, and 10 benign tumor diagnoses. Of the 90 patients with ccRCC, 15 had a past medical history of cancer or chronic kidney disease, and were subsequently excluded from the study. Of the remaining 75 patients, eight had metastatic ccRCC. The clinicopathological information of 67 patients with non-metastatic ccRCC is shown in Table 2. The group consisted of 48 (71.6%) males and 19 (28.4%) females. Seven (10.4%) patients were under the age of 40, 14 (20.9%) were in their 40s, 19 (28.4%) were in their 50s, 18 (26.9%) were in their 60s, and nine (13.4%) were over the age of 70. Regarding pT stage, 53 (79.1%), five (7.4%), three (4.5%), and six (9.0%) patients were characterized with pT1a, pT1b, pT2a, and pT3a, respectively. No metastasis to the lymph nodes (N0) was observed in any patients. Therefore, N stage is not described in the current study. Based on the RCC staging system, 58 (86.6%), 2 (3.0%), and 7 (10.4%) patients were characterized with stage I, II, and III kidney cancer, respectively. According to the Furman grading or the WHO/ISUP grading system, 4 (6.0%), 36 (53.7%), 24 (35.8%), and 3 (4.5%) patients were classified as having grade I, II, III, and IV ccRCC, respectively.

There were ten patients in the benign control group which included six cases of angiomyolipoma, two cases of oncocytoma, and one case each of simple cortical cyst and pseudocyst. Eight benign tumors had a maximal diameter of less than 4 cm. The largest benign tumor was 8 cm, which was observed in the patient with the pseudocyst. The median age of the benign control group was 57.7 years.

Table 2. Clinicopathological data of 67 patients with non-metastatic clear cell renal cell carcinoma

Variable		**n (%)**
Sex		
	Male	48 (71.6)
	Female	19 (28.4)
Age		
	<40	7 (10.4)
	40–49	14 (20.9)
	50–59	19 (28.4)
	60–69	18 (26.9)
	≥70	9 (13.4)
Pathological T stage		
	T1a	53 (79.1)
	T1b	5 (7.4)
	T2a	3 (4.5)
	T3a	6 (9.0)
N stage		
	N0	67 (100.0)
Stage		
	I	58 (86.6)
	II	2 (3.0)
	III	7 (10.4)
Grade		
	I	4 (6.0)
	II	36 (53.7)
	III	24 (35.8)
	IV	3 (4.5)
Total, n (%)		67 (100.0)

2. Circulating-tumor DNA characteristics

The median amounts of extracted cfDNA were 46.0 ng and 29.9 ng in metastatic non-metastatic and ccRCC, respectively, with 29.9 ng and 30.2 ng , extracted from pT1a and p1b-3a non-metastatic ccRCC, respectively. The amount of input DNA for library preparation ranged from 6.1 ng to 30.1 ng, except for one pT1a sample of 4.5 ng. The median amounts of input DNA for library preparation were 16.0 ng for pT1a and 16.8 ng for pT1b-3a non-metastatic ccRCC, and 26.8 ng for metastatic ccRCC. The median average depth per patient was 18,708× for metastatic ccRCC and 20,356× for non-metastatic ccRCC, with 19,946× and 22,482× for T1a and T1b-3a non-metastatic ccRCC, respectively.

Figure 3 shows the genomic landscape of the ctDNA of patients with non-metastatic ccRCC. The middle heat map shows events of ctDNA detection with their percentages in the total patient population on the right. ctDNA was detected in 18 (26.9%) of 67 patients. The top histogram indicates the number of ctDNA variants detected per patient. The maximum number of ctDNA varients detected per patient was two. The right histogram indicates the number of ctDNA variants detected based on the gene. *NF1* was the most frequently ctDNA-detected gene, followed by *TP53,* and *KDM6A,* respectively. *EGFR*, *KDM5A, MET, PBRM1, PIK3CA,* and *PTEN* were detected twice, and *ARID1A*, *SETD2,* and *VHL* were detected once. No ctDNA was observed in *BAP1*, *EGFR*, *ELOC*, *KDM5C,* or *NF2* genes. Colors in the middle heat map, and the histograms on the top and right indicate variant classifications. Missense was the most frequently observed mutation. The bottom heat map shows information on pathological T stage and tumor grade per patient.

Figure 4 shows genomic landscape of ctDNA from patients with non-metastatic and metastatic ccRCC. ctDNA was detected in 24 (32.0%) out of 75 patients. The maximum number of ctDNA variants per patient was five. *NF1* was the most frequently ctDNA-detected gene, followed by *PBRM1, PTEN, TP53,* and *VHL,* which were detected once each. Missense was the most frequently

observed mutation. The bottom heat map shows information on clinicopathological data per patient (pT, tumor grade, LVI, FTI and VTT).

Clinicopathological features of non-metastatic ccRCC patients with ctDNA are shown with information about ctDNA in Table 3. Patients were sorted in ascending order according to tumor size. A total of 21 ctDNA variants were detected from 18 patients. One ctDNA *VHL* variant was found in a patient with a 3.8 cm grade IV tumor. This ccRCC showed sarcomatoid differentiation with tumor necrosis. Two ctDNA *PBRM1* variants were found, of which one was found in a patient with a 3.8 cm grade II tumor, and the other one was detected in a patient with a 6.9 cm grade III tumor. Three *TP53* variants were detected in samples from two patients with grade III and one patient with grade I tumors. Two of the three patients were over 60 years of age.

Clinicopathological features of patients with metastatic ccRCC with ctDNA are shown with information about ctDNA variants in Table 4. Patients were sorted in ascending order according to primary tumor size. A total of 18 ctDNA variants were detected from six patients. ctDNA *VHL* variants were detected in four patients, and ctDNA *TP53* variants were detected in two patients. All ctDNA *MTOR*, *PBRM1*, *PTEN,* and *SETD2* variants were accompanied by ctDNA variants of *VHL*. Two patients with ctDNA had grade IV tumors, one of which showed sarcomatoid differentiation with tumor necrosis.

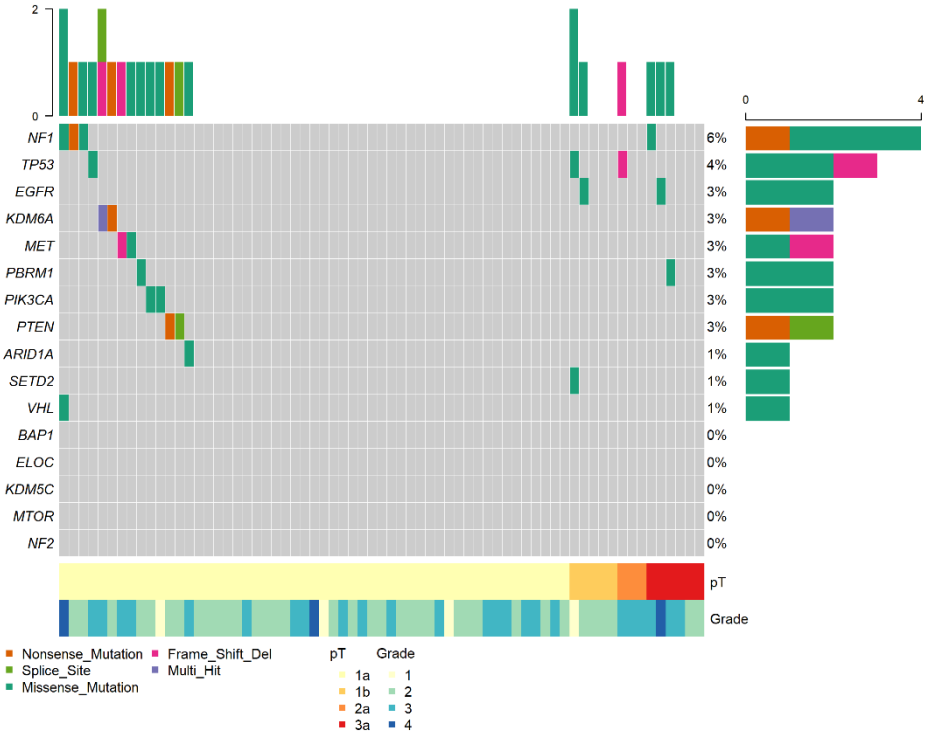

Figure 3. Genomic landscape of circulating-tumor DNA in 67 patients with non-metastatic clear cell renal cell carcinoma

Middle heat map shows ctDNA detection events with their percentages in the total patients on the right. Top histogram shows the number of ctDNA variants per sample; Right histogram shows the number of ctDNA per gene; Bottom heat map shows information about the pathological T stage and tumor grade per sample.

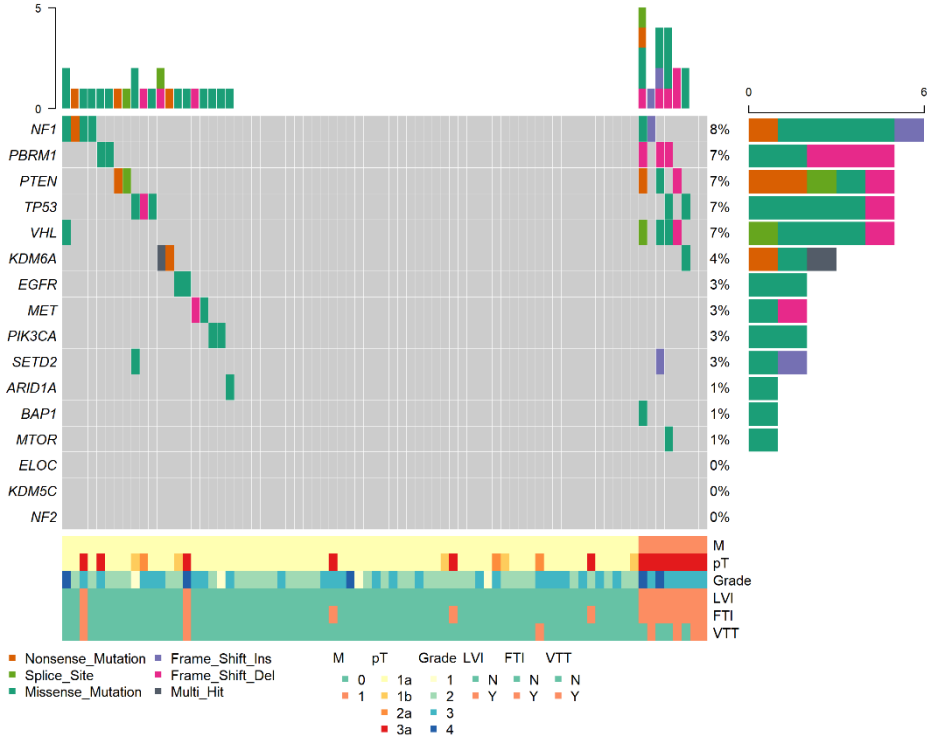

Figure 4. Genomic landscape of circulating-tumor DNA in non-metastatic and metastatic clear cell renal cell carcinoma

Middle heat map shows ctDNA detection events with their percentages in the total patients on the right. Top histogram shows the number of ctDNA variants per sample. Right histogram shows the number of ctDNA per gene. Bottom heat map showing clinicopathological information per sample.

Abbreviations: FTI, fat tissue invasion; LVI, lymphovascular invasion; M, metastasis; VTT, venous tumor thrombus

Table 3. Clinicopathological features and circulating-tumor DNA in non-metastatic clear cell renal cell carcinoma

ID	Sex	Age	Tumor size (cm)	pT	Stage	Grade	ctDNA variant		%VAF
098	M	56	1.2	1a	I	I	*PIK3CA*	Q1033L	0.115
004	F	50	1.9	1a	I	II	*PTEN*	Y16X	0.499
101	F	71	1.3	1a	I	II	*KDM6A*	Q367X	0.216
057	M	56	1.7	1a	I	III	*TP53*	R196Q	0.453
059	M	59	2.2	1a	I	II	*NF1*	K513X	0.35
083	F	62	3.0	1a	I	III	*KDM6A*	Splicing	0.352
							KDM6A	E1335fs	0.614
007	M	63	2.5	1a	I	II	*PTEN*	Splicing	2.749
118	F	51	2.8	1a	I	III	*MET*	R987Q	0.378
121	M	62	3.8	1a	I	IV*	*VHL*	M1I	0.283
							NF1	Q1447H	0.139
069	M	68	3.8	1a	I	II	*PBRM1*	R710Q	0.236
108	F	72	2.8	1a	I	II	*NF1*	E1436K	0.456
025	M	47	4.0	1a	I	II	*PIK3CA*	S235F	0.232
036	M	79	4.1	1b	I	II	*EGFR*	A647T	0.141
020	F	64	5.4	1b	I	I	*TP53*	Y220C	0.442
							SETD2	P2361H	0.421
094†	M	59	5.5	3a	III	III	*NF1*	P2221L	0.167
015	M	63	6.9	3a	III	III	*PBRM1*	Y1506H	0.489
048	M	79	8.0	2a	II	III	*TP53*	P153fs	0.178
058†	M	86	9.5	3a	III	IV	*EGFR*	R958C	0.191

* Sarcomatoid differentiation with tumor necrosis

† Lymphovascular invasion, fat tissue invasion, and venous tumor thrombus were identified in two patients.

Abbreviations: F, female; M, male; VAF, variant allele frequency

Table 4. Clinicopathological features and circulating-tumor DNA in metastatic clear cell renal cell carcinoma

ID	Sex	Age	Tumor size (cm)	pT	LVI	FTI	VTT	Gradect	DNA variant		%VAF
062	M	52	6.8	3a	Y	Y	N	III	*MTOR*	C1483R	15.627
									PBRM1	F1100fs	6.66
									TP53	G245C	6.268
									VHL	L169P	9.489
093	F	71	7.0	3a	Y	Y	Y	III	*NF1*	S1497fs	0.263
104	M	60	9.0	3a	Y	Y	N	IV*	*PBRM1*	P1272fs	5.235
									PTEN	R130X	6.661
									VHL	Splicing	4.437
									BAP1	R114H	0.257
									NF1	D1849E	0.104
088	M	81	11.0	3a	Y	Y	N	III	*TP53*	C238Y	1.126
									KDM6A	L361F	0.737
032	M	52	12.5	3a	Y	Y	Y	III	*PTEN*	K128fs	1.526
									VHL	R69fs	1.21
019	M	69	17.0	3a	Y	Y	Y	IV	*PBRM1*	K907fs	0.37
									PTEN	R173P	0.595
									SETD2	L1923fs	0.907
									VHL	L128P	0.733

* Sarcomatoid differentiation with tumor necrosis

Abbreviations: F, female; FTI, fat tissue invasion; LVI, lymphovascular invasion; M, male; N, no; VAF, variant allele frequency; VTT, venous tumor thrombus; Y, yes

3. Detection rate of circulating-tumor DNA

Figure 5 shows the trend of detection rates of ctDNA by group. The detection rate increased from pT1a to M1 groups, except for the pT2a group. The number of patients with pT1a pT stage was highest. ctDNA was detected in 18 of the 67 patients with non-metastatic ccRCC, resulting in an overall ctDNA detection rate of 26.9 %. ctDNA was detected in 12 of the 53 patients with pT1a (22.6%). The ctDNA detection rates in patients with pT1b, pT2a, or pT3a were 40.0%, 33.3%, and 50.0%, respectively. Taken together, ctDNA was detected in six (42.9%) of the 14 patients with pT1b-3a. ctDNA detection rates between pT1a and pT1b-3a pT stages were not significantly different ($p = 0.236$). ctDNA was detected in six (75.0%) of the eight patients with metastatic ccRCC. The detection rate of metastatic ccRCC was significantly higher than that of pT1a ($p = 0.009$), but no significant difference between pT1b-3a ccRCC and metastatic ccRCC was observed ($p = 0.312$). Table 3 summarizes and compares the detection rates of ctDNA in non-metastatic and metastatic ccRCC. Non-metastatic ccRCC was further evaluated according to pT stage. Among the patients with benign tumors, one patient with a large pseudocyst was identified as having a somatic variant of uncertain significance (R426H of *MET*).

4. Variant allele frequency of circulating-tumor DNA

Figure 6 shows the VAFs of ctDNA according to group. The median VAF of non-metastatic ccRCC was 0.35%, which was significantly lower than that of metastatic ccRCC (1.168%, $p < 0.001$). The median VAFs of non-metastatic pT1a and pT1b-3a were 0.351% and 0.191%, respectively, which was significantly lower than those of metastatic ccRCC ($p = 0.002$ and 0.003, respectively). However, no significant difference was observed between pT1a and pT1b-3a non-metastatic ccRCC ($p = 0.400$). Table 5 summarizes and compares the VAFs of ctDNA in non-metastatic and metastatic ccRCC. Non-metastatic ccRCC was further evaluated according to pT.

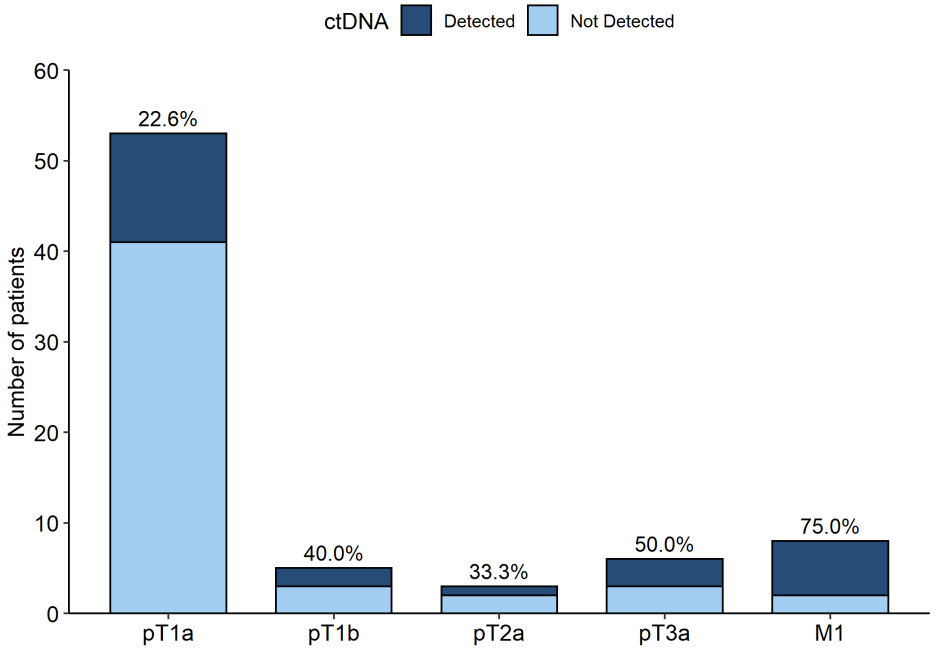

Figure 5. Detection rate of circulating-tumor DNA based on pathological T stage and metastasis

Barplots of the ctDNA detection rate based on pathological T stage (pT) stage and metastasis. The x-axis indicates pathological stage (pT1a, pT1b, pT2a, pT3a, and M1), the y-axis indicates the number of samples in each group. ctDNA detection rates in non-metastatic ccRCC were 22.6%, 40.0%, 33.3%, and 50.0% in the pT1, pT1b, pT2a, and pT3a groups, respectively, while the ctDNA detection rate in metastatic ccRCC was 75.0%.

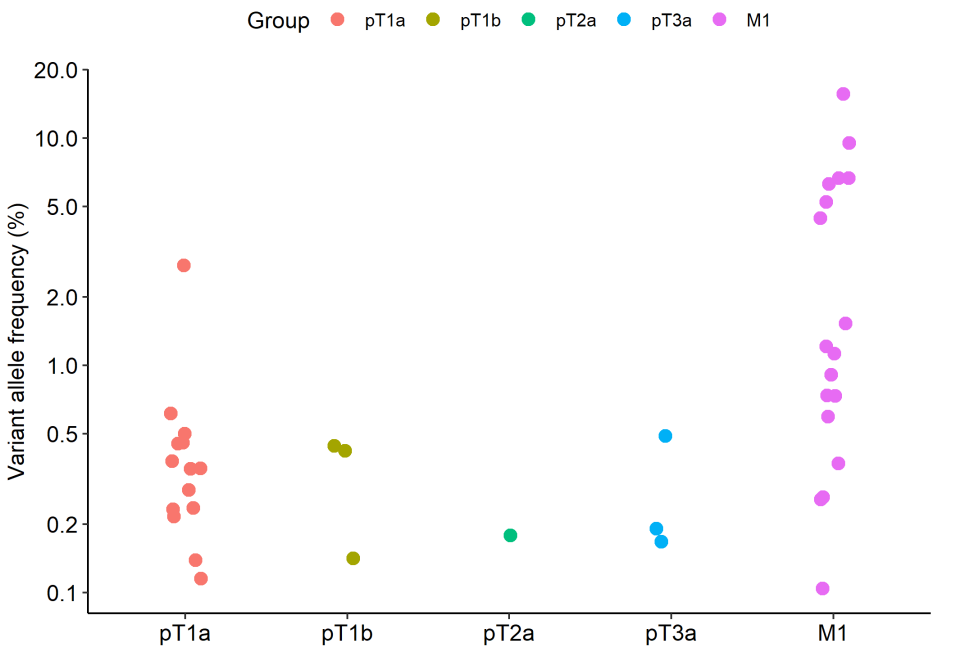

Figure 6. Variant allele frequency of circulating-tumor DNA based on pathological T stage and metastasis

A strip chart of VAFs of ctDNA based on pathological T stage and metastasis. The x-axis indicates categories of stage (pT1a, pT1b, pT2a, pT3a, and M1), the y-axis indicates the VAF of ctDNA for each group. The median VAFs were 0.351%, 0.421%, 0.178%, and 0.191% in the pT1, pT1b, pT2a, and pT3 groups respectively, while the median VAF in metastatic ccRCC was 1.168%.

Abbreviation: VAF, variant allele frequency

Table 5. Detection rates and variant allele frequencies of circulating-tumor DNA

	Group				*p*-value			
	pT1a	pT1b-3a	M0	M1	pT1a vs. pT1b-3a	pT1a vs. M1	pT1b-3a vs.M1	M0 vs. M1
Detection rate (%)	22.6	42.9	26.9	75.0	0.239	0.009	0.312	0.018
Median variant allele frequency (%)	0.351	0.191	0.350	1.168	0.400	0.002	0.003	< 0.001

5. Proportion of genes with circulating-tumor DNA

Figure 7 shows the proportion of genes with ctDNA in non-metastatic ccRCC compared with metastatic ccRCC. Genes were ordered from left to right by the number of patients with ctDNA in metastatic ccRCC. The proportion of specific genes with ctDNA was calculated by dividing the number of ctDNA detected in a specific gene by the total number of detected ctDNA variants. The proportion of each gene is described at the top of each bar. In non-metastatic ccRCC, *NF1* was the most frequently detected gene, observed in four (16.7%) of the 21 ctDNA variants detected. The proportion of genes with ctDNA was 14.3% (3/21) for both *TP53* and *KDM6A*. The proportion of genes with ctDNA was 4.8% (1/21) for both *VHL* and *SETD2*. *VHL* was the most frequently detected gene in metastatic ccRCC, observed in four (55.6%) of the 18 ctDNA variants detected. The proportion of genes with ctDNA was 16.7% (3/18) for both *PTEN* and *PBRM1*. The proportion of genes with ctDNA was 11.1% (2/18) for both *TP53* and *NF1*.

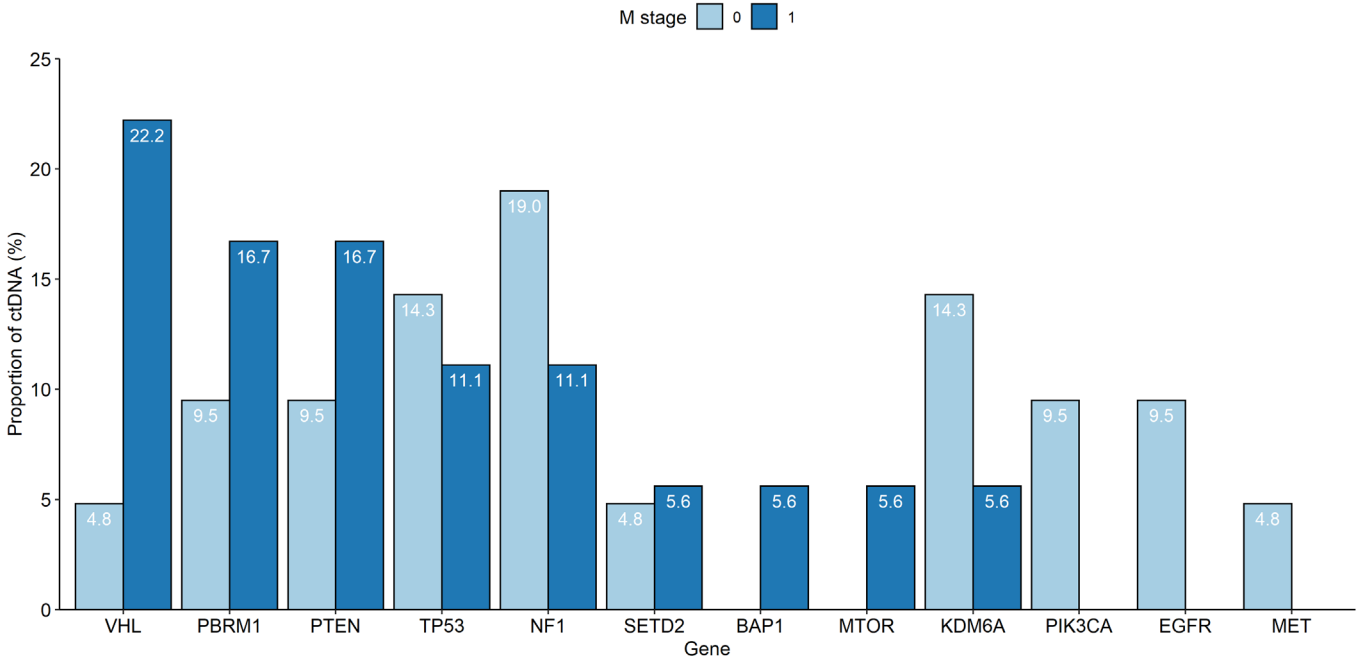

Figure 7. Proportion of genes with circulating-tumor DNA

Proportion of ctDNA was calculated as the number of patients with ctDNA detected for each gene divided by the total number of patients. *NF1* was the most frequently detected gene in non-metastatic ccRCC, followed by *KDM5A,* and *TP53*, respectively. *VHL* was the most frequently detected gene in metastatic ccRCC, followed by *PBRM1,* and *PTEN*, respectively.

6. Relationship between circulating-tumor DNA and clinicopathological features of non-metastatic clear cell renal cell carcinoma

Tumor size was compared between ctDNA detection and non-detection groups (Figure 8). The relationship between the ctDNA detection group and non-detection group in non-metastatic ccRCC was evaluated. The relationship between ctDNA and the primary tumor size in metastatic ccRCC was also examined and compared with that of non-metastatic ccRCC. Tumor size in non-metastatic ccRCC was further investigated in relation to ctDNA after stratifying by pT stage into pT1a and pT1b-3a groups. In non-metastatic ccRCC, for all patients, the median tumor size was 2.2 cm. The median tumor size in the ctDNA detection group was significantly larger than that of non-detection group (3.4 cm v 2.0 cm, respectively; $p = 0.014$). In contrast, there was no significant difference in the primary tumor median size in metastatic ccRCC in the ctDNA detection and non-detection (10.0 cm v 8.5 cm, respectively; $p = 0.64$). It should be noted that only eight patients were involved when comparing the primary tumor size between ctDNA detection and non-detection groups. The median tumor size of the pT1a groups was larger in the ctDNA detection group than in the non-detection group (2.7 cm v 1.8 cm; $p = 0.053$). However, the relationship between median tumor size and ctDNA detection was not significant for the pT1b-3a group (ctDNA detection v non-detection, 6.2 cm v 6.5 cm, respectively; $p > 0.999$).

ctDNA detection according to tumor grade is shown in Figure 9. The tumor grade was grouped into grade I/II and grade III/IV. There was no relationship observed between ctDNA detection and tumor grade in non-metastatic ccRCC ($p = 0.781$), including by pT stage (pT1a, $p > 0.999$; pT1b-3a, $p = 0.592$). The relationship between tumor grade and ctDNA could not be evaluated in the metastatic ccRCC group because all tumors were identified to be grade III and IV.

Figure 10 shows the relationship between ctDNA and poor prognostic features

(LVI, FTI, and VTT) in the non-metastatic ccRCC group. LVI, FTI, and VTT are known as poor prognostic factors in ccRCC.[36-38] Although no apparent relationships were observed between ctDNA detection and poor prognostic features, due to the insufficient number of non-metastatic ccRCC patients with LVI, FTI, or VTT (only six patients included), ctDNA was more frequently observed when LVI, FTI, and VTT were positive. Of note, two patents with LVI also showed FTI and VTT, and ctDNA was detected in both patients.

Figure 11 shows a comparison of patient age between ctDNA detection and non-detection groups in non-metastatic ccRCC. The median age of the ctDNA detection group was significantly greater than that of the ctDNA non-detection group (62.5 years v 53.0 years, $p = 0.002$). Because CH-related variants were more frequently detected in the elderly, we additionally investigated the association between CH-related variants of ctDNA and patient age. *TP53* and *KMD6A* were the CH-related genes included in the targeted next-generation sequencing (NGS) panel used in the present study. The proportion of CH-related genes in ctDNA was 36.4% for those aged 60 years or older, and 14.3% for those aged under 60 years, which was not significantly different ($p = 0.631$).

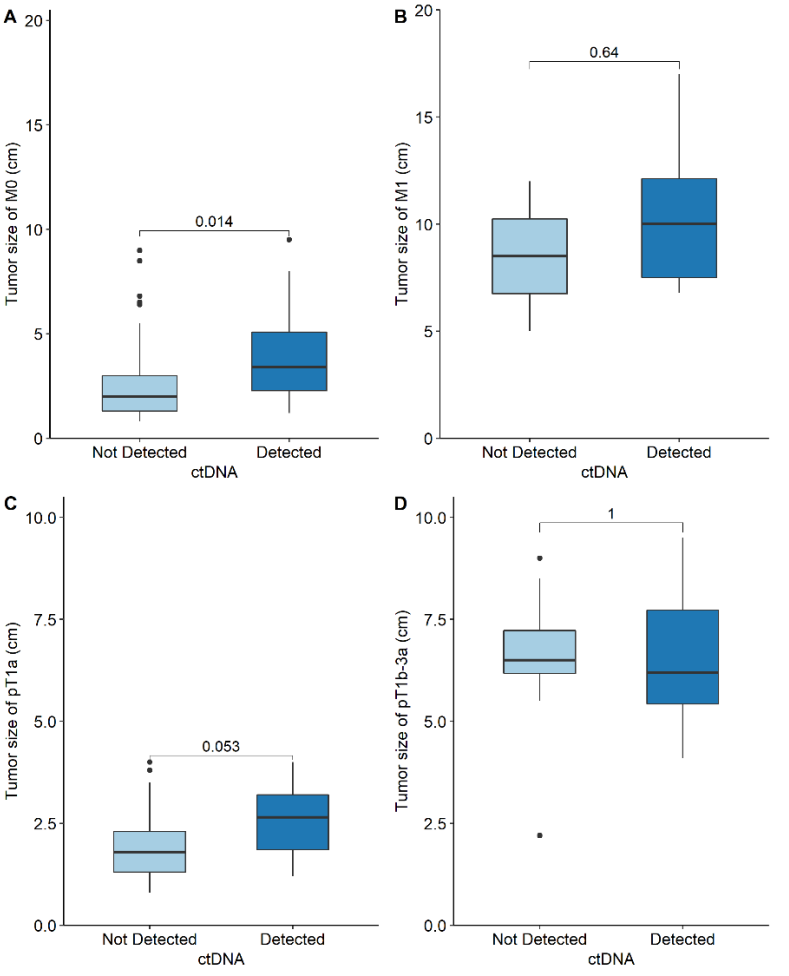

Figure 8. Comparison of tumor size according to circulating-tumor DNA

(A) Comparison of tumor size between ctDNA detection and non-detection groups in patients with non-metastatic ccRCC. Tumor size was significantly larger when ctDNA was detected (p = 0.014). (B) Comparison of tumor size in patients with metastatic ccRCC without and with ctDNA detected. No significant difference was observed (p = 0.640). (C) Comparison of tumor size in patients with pT1a ccRCC without and with ctDNA detected. Tumor size tended to increase in pT1a ccRCC when ctDNA was detected (p = 0.053). (D) Comparison of tumor size between groups with pT1b-3a ccRCC without and with ctDNA detected. No significant difference was observed (p > 0.999).

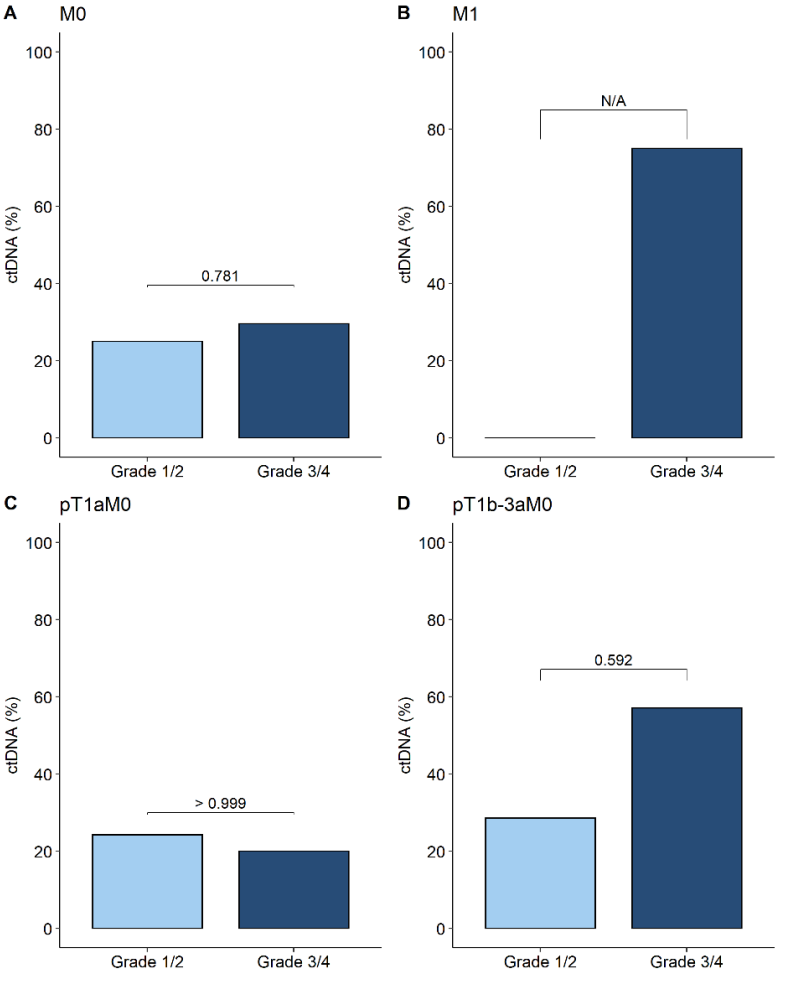

Figure 9. Relationship between circulating-tumor DNA and tumor grade

(A) ctDNA detection according to tumor grade in patients with non-metastatic ccRCC. No significant difference was observed ($p = 0.781$). (B) ctDNA detection according to tumor grade in patients with metastatic ccRCC. All metastatic ccRCC showed tumor grade III and IV. (C) ctDNA detection according to tumor grade in patients with pT1a ccRCC. No significant difference was observed ($p > 0.999$). (D) ctDNA detection according to tumor grade in patients with pT1b-3a ccRCC. No significant difference was observed ($p = 0.592$).

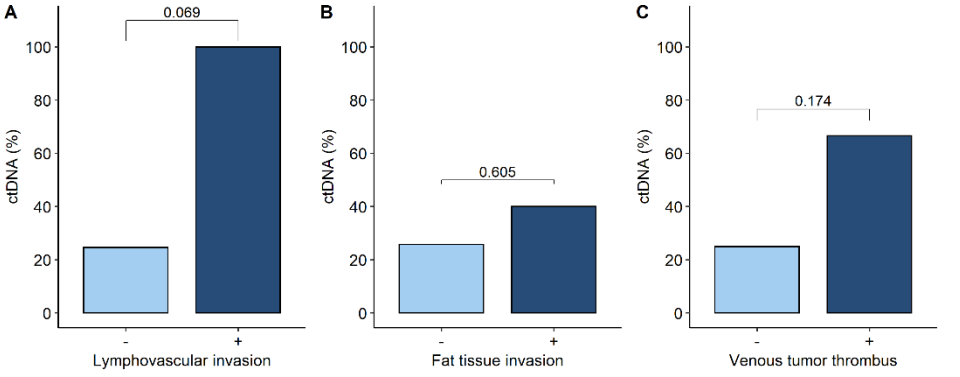

Figure 10. Relationship between circulating-tumor DNA and poor prognostic features of non-metastatic clear cell renal cell carcinoma

(A) ctDNA was more frequently detected when lymphovascular invasion was observed (p = 0.069). (B) ctDNA was slightly more frequently detected when fat tissue invasion was positive (p = 0.605). (C) ctDNA tended to be more frequently detected when venous tumor thrombus was positive (p = 0.174). The statistical differences between groups could not be accurately evaluated due to the small number of non-metastatic patients with poor prognostic features.

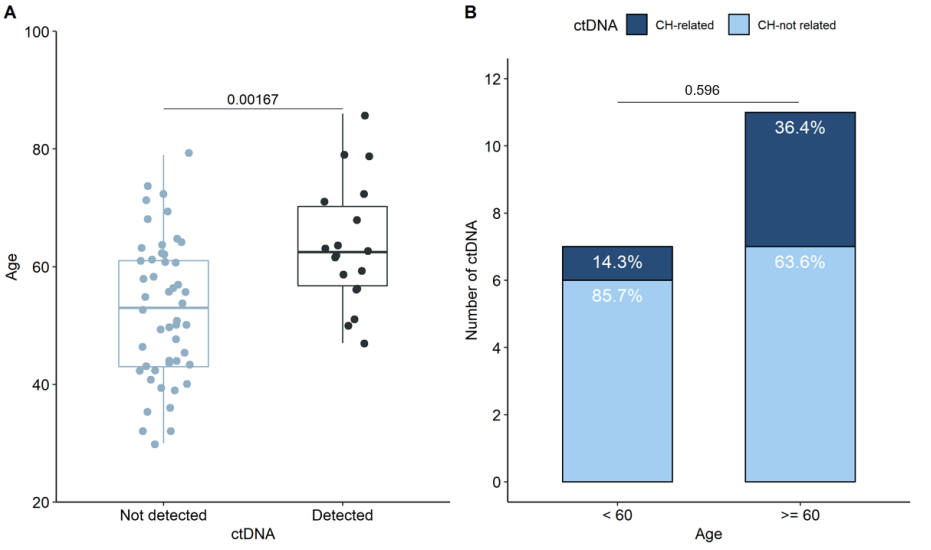

Figure 11. Relationship between ctDNA and patient age in non-metastatic clear cell renal cell carcinoma

(A) Comparison of patient age between ctDNA detection and not detection groups in non-metastatic ccRCC. Patients with ctDNA detected were significantly older than patients without ctDNA ($p = 0.00167$). (B) The proportion of patients with non-metastatic ccRCC with ctDNA of CH-related genes (*TP53* and *KDM6A*). Eleven patients who were over 60 years of age had at least one ctDNA variant. Of these 11 patients, four (36.4%) had one or more ctDNA variant of CH-related genes. Seven patients who were under 60 years of age had at least one ctDNA variant. Of these seven patients, one (14.3%) had ctDNA variant of CH-related genes. The proportions of CH-related genes between the two groups was not significantly different ($p = 0.596$).

7. Positive concordance of somatic variants between circulating-tumor DNA and matched tissue

The positive concordance of somatic variants between ctDNA and matched tissue in ccRCC is shown in Figure 12. Eight patients with ccRCC who had available tumor tissue for NGS were selected. Of them, five patients had non-metastatic ccRCC, two patients had metastatic ccRCC, and one patient had a benign renal tumor. Positive concordant somatic variants were detected in two of the eight patients, resulting in an overall concordance rate of 25.0%. Two patients were identified as having metastatic ccRCC. No concordant variant was observed in patients with non-metastatic ccRCC. One patient with metastatic ccRCC did not show a somatic variant of *VHL* in tissue but ctDNA of *VHL* was observed in the plasma. Somatic variants of *VHL* were detected in the tissue of three of the five patients with non-metastatic ccRCC, however, no concordant somatic variant was observed in plasma. No somatic variant was detected in the tissue of two of the five patients with non-metastatic ccRCC, but ctDNA was observed in plasma.

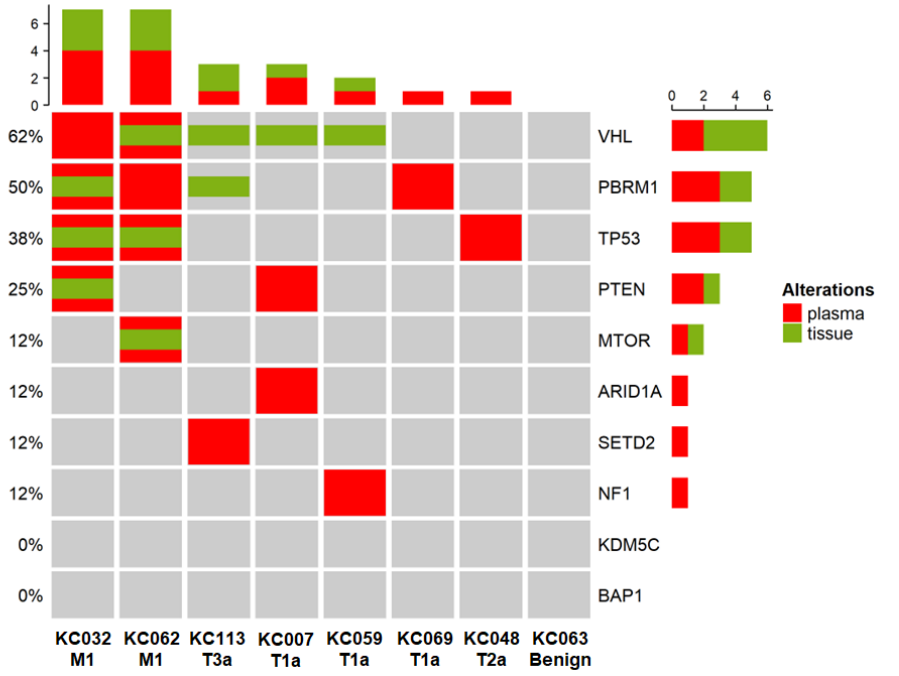

Figure 12. Positive concordance of somatic variants between circulating-tumor DNA and matched tissue samples in clear cell renal cell carcinoma

Eight patients who had available tumor tissue for next-generation sequencing were selected. Stage is shown at the bottom of the heatmap. The heatmap indicates where each somatic variant was detected in each gene. Red indicates somatic variants detected in plasma, while green indicates somatic variants detected in tissue. The top histogram shows the number of somatic variants detected per patient. The right histogram shows the number of somatic variants detected per gene, with each ratio indicated on the left of the heatmap.

8. Example case of recurrence after partial nephrectomy for non-metastatic clear cell renal cell carcinoma with circulating-tumor DNA

A 62-year-old male with a medical history of diabetes mellitus and hypertension visited the outpatient clinic for a small renal mass discovered incidentally on an imaging study. The heterogeneous enhancing mass was about 3.4 cm in diameter and situated on the mid pole of the right kidney (Figure 13A). Partial nephrectomy was performed because small-sized RCC was suspected. The renal mass was excised, and complete removal was confirmed by postoperative magnetic resonance imaging (Figure 13B). Pathological examination revealed a WHO/ISUP nuclear grade IV, 3.8 cm ccRCC tumor. Focal necrosis (30%) and sarcomatoid differentiation (10%) were observed. The tumor was limited in the kidney and no lymphovascular invasion was observed. Somatic variants of *VHL* and *NF1* were detected by ctDNA analysis. Sixteen months after surgery, a 3.8 cm mass-like lesion at the prior surgical site was observed, suggesting ccRCC recurrence (Figure 13C). Radical nephrectomy was performed, and ccRCC recurrence was confirmed on pathological examination. The maximum tumor diameter was 5.4 cm and the WHO/ISUP nuclear grade was grade III. Renal sinus fat tissue, renal vein, and lymphovascular invasion was observed.

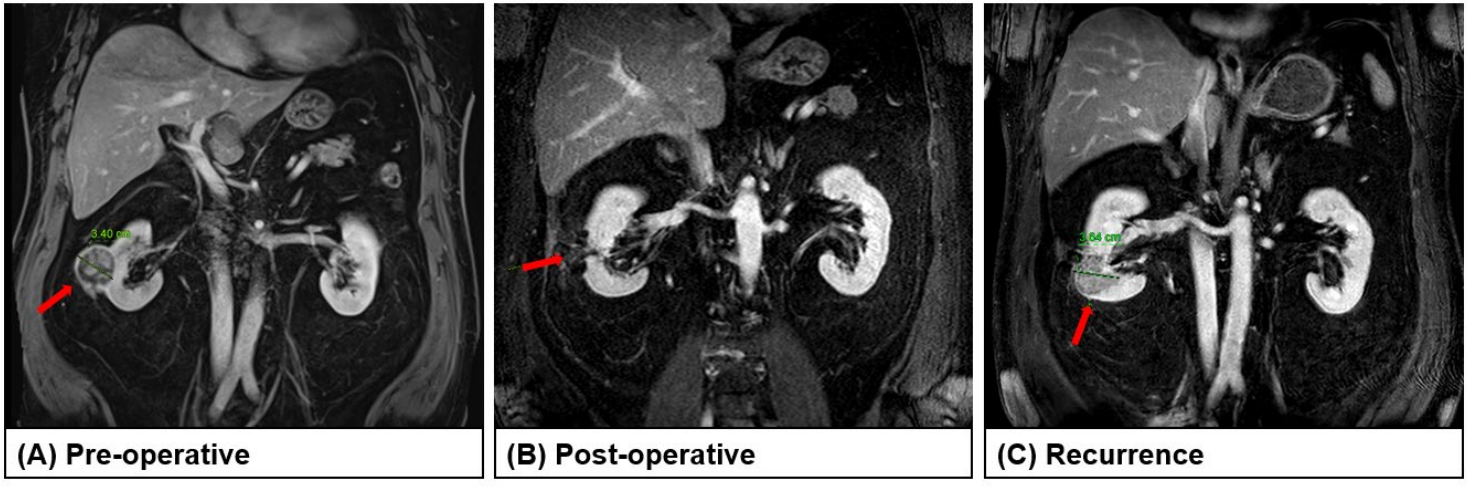

Figure 13. A case of clear cell renal cell carcinoma recurrence after partial nephrectomy in a patient with circulating-tumor DNA

(A) Pre-operative magnetic resonance imaging (MRI) shows a heterogeneous enhancing mass about 3.4 cm on the mid pole of the right kidney. (B) Postoperative MRI shows a partial nephrectomy state. ccRCC is completely removed. (C) Sixteen months after the surgery, MRI shows 3.8 cm mass-like lesion at the previous surgical site, suggesting ccRCC recurrence.

IV. DISCUSSION

ctDNA analysis has been performed for diagnosis, monitoring, prognosis prediction, and treatment selection of various cancers.[18] For example, using ctDNA assay for the detection of *EGFR* somatic variants in non-small cell lung cancer and detection of *KRAS* somatic variants in metastatic colorectal cancer is now well-established, and the clinical utility of ctDNA detection of somatic variants for the diagnosis, treatment, monitoring, and selection of target agent has been demonstrated.[39,40] In addition, analytically acceptable detection rates of ctDNA have been reported for colorectal, breast, lung, pancreas, liver, and ovarian cancers.[41,42] Risk stratification can be determined using pre- and postoperative ctDNA assays to predict recurrence and survival in patients with cancer, which facilitates decision making regarding appropriate postoperative management including use of adjuvant therapies.[43,44]

Similarly, several ctDNA analysis studies of RCC have investigated mutational profiles of ctDNA and report their concordance with matched tissue.[19-21] The role of ctDNA in predicting prognosis, treatment response, and resistance for RCC has also been investigated.[19,21,27] However, the majority of these studies were conducted on metastatic or large-sized ccRCC. Few studies have examined the characteristics of ctDNA analysis in non-metastatic ccRCC, particularly for small-sized ccRCC. Table 6 shows that the present study includes a greater number of patients with small-sized non-metastatic ccRCC compared to those included in previous studies using next-generation sequencing technology.[19-21] Of note, a substantial number of ccRCC categorized as pT1a were included in the present study. Therefore, the current study contributes to further elucidating the characteristics of ctDNA in small non-metastatic ccRCC.

Table 6. Comparison of the detection rates of circulating-tumor DNA in clear cell renal cell carcinoma observed in previous studies versus the present study

Reference	Total, n	Non-metastatic			Metastatic			Comment
		Patient, n (%)	Median tumor size (cm)	ctDNA detection, n (%)	Patient, n (%)	Median tumor size (cm)	ctDNA detection, n (%)	
Yoshiyuki Yamamoto (2019)[19]	53	14 (26.4)	–	2 (14.3)	39 (73.6)	–	14 (35.9)	
Christopher G. Smith (2020)[20]	29	24 (82.8)	6.1	14 (48.3)	5 (17.2)	7.4	4 (80.0)	Cohort DIAMOND
Christopher G. Smith (2020)[20]	35	–	–	–	35 (100.0)	7.75	9 (25.7)	Cohort MonRec
Yeon Jeong Kim (2021)[21]	18	10 (55.6)	7.1	4 (40.0)	8 (80.0)	10.0	7 (50.0)	
Present study	75	67 (89.3)	2.2	18 (26.9)	8 (10.7)	10.0	6 (75.0)	

Detection rates of ctDNA in ccRCC vary depending on the study. Detection rates of ctDNA in non-metastatic ccRCC have not been clearly established. Although a few studies report a ctDNA detection rate of non-metastatic ccRCC in the range of 14.3-48.3%, the results of those studies are questionable due to limited sample sizes. Moreover, the median size of the primary tumors of non-metastatic ccRCC in these studies were greater than 4 cm (Table 6).[20,21] In the present study, the detection rate of ctDNA in non-metastatic ccRCC was 26.9%, which is reliable due to the relatively large sample size. The detection rate of non-metastatic ccRCC was low compared with those reported for other cancers such as colorectal, breast, lung, and ovarian cancer, which have detection rates of 59-71% in the non-metastatic state. However, considering that only 20% of the non-metastatic cancers investigated were classified as stage T1, the detection rate of non-metastatic ccRCC in this study seemed acceptable.[41] In metastatic ccRCC, detection rates of ctDNA have been reported to range from 25.7-80.0%. Despite the small sample size in the present study, the detection rate of ctDNA in metastatic ccRCC was 75.0%, which was consistent with previous findings.[19-21]

Considering that the amount of ctDNA in plasma correlates with tumor size, the relatively small tumor sizes observed in the present study (primarily less than 4 cm), may contribute to the low ctDNA detection rate observed here. According to a previous report, the fraction of ctDNA was less than 0.1% in the peripheral blood when the tumor size was 2.4 cm in diameter.[45] To increase the sensitivity of ctDNA analysis, an increase in input plasma volume may be attempted.[46] However, collecting a larger volume of blood is not always realistic in the clinical setting, and the use of more plasma does not always result in a proportional increase in the amount of cfDNA.[47,48] In addition to the size of the tumor, the type of cancer significantly affects the amount of ctDNA in plasma. The amount of ctDNA released into the bloodstream is determined by the characteristics of the cancer, such as tumor vascularization and histological type. RCC has been

classified as a low-ctDNA cancer by several studies, but the cause of the low ctDNA level has not been clearly established.[20,46,49]

In the current study, the proportion of genes with ctDNA in non-metastatic ccRCC was different from the mutational profiles in tissue. Although the frequency reported in the literature varies depending on the study, the most frequently mutated gene in ccRCC tissue is *VHL*, followed by *PBRM1*, *SETD2*, *BAP1,* and *KDM5C*.[28,50] In contrast to the proportion of genes in metastatic ccRCC, only one somatic variant of *VHL* was detected in non-metastatic ccRCC in the current study. Low numbers of *PBRM1*, the second most common mutated genes in tissue, were detected in non-metastatic ccRCC.[28,50] Considering that *VHL* is considered to be an early evolutionary ancestor gene and *PBRM1* is highly involved in the early evolution of ccRCC, it was unexpected to find that these core gene mutations were less frequently detected in non-metastatic ccRCC.[50] Interestingly, the proportion of ctDNA of *NF1*, *TP53,* and *KDM6A* was high in non-metastatic ccRCC.

Variants of *NF1* are infrequently observed in ccRCC tissue.[50,51] However, one study which analyzed ctDNA in metastatic ccRCC showed that *NF1* was one of the most frequent genes with ctDNA, accounting for 16% of total genomic alterations.[27] Moreover, somatic variants of *NF1* can be found in a wide range of malignancies and have been recognized as possible drivers in multiple cancers.[52] Similarly, a large scale pan-cancer ctDNA analysis categorized *NF1* as a common driver gene in many cancers.[53] *TP53* variants have been commonly detected in numerous studies and are recognized as one of the drivers in ccRCC. The variant frequency of *TP53* is relatively low compared with *VHL*, *PBRM1*, *SETD2,* and *BAP1*. *TP53* variants are also known to be observed in a critical subclonal event.[50,54] A genomic meta-analysis of ccRCC demonstrated that the ctDNA of *TP53* is more prevalent in metastatic sites of ccRCC.[55] In addition, several ctDNA studies showed that *TP53* is one of the most frequent ctDNA-related genes in metastatic RCC.[56] Based on the previous reports, ctDNA of *TP53* may be a

potential biomarker for predicting poor prognosis in non-metastatic ccRCC. *KDM6A* is a histone modifier and variants have been detected in 1% of ccRCC. The clinical significance of ctDNA of *KDM6A* is uncertain because there is little information about *KDM6A* variants reported in ctDNA studies.

ctDNA analysis is emerging as a tool for prognosis prediction and risk stratification, and it may reflect clinicopathological features of ccRCC.[45] In the present study, the most critical factor associated with ctDNA detection was a metastatic event at diagnosis, implying that ctDNA detection is associated with reduced survival rates.[57,58] alone study report that ctDNA detection was associated with decreased progression-free survival and cancer-specific survival in patients with metastatic ccRCC.[19]

Tumor size is another important prognostic factor of ccRCC.[57] A recent study showed that larger tumor volume of localized ccRCC adversely affected clinical outcomes.[59] The current study demonstrated that the tumor size was larger when ctDNA was detected in non-metastatic ccRCC. Specifically, this relationship was observed in pT1a ccRCC, implying that ctDNA analysis can be used for risk stratification in patients with small renal masses. In pT1b-3a ccRCC, no relationship between ctDNA and tumor size was observed. Although statistical power seemed limited due to the small number of patients with this pathological T stage, there may be other critical factors, such as LVI, FTI, and VTT, that can affect ctDNA detection in larger-sized non-metastatic ccRCC.[36-38] In addition, considering that there was no significant difference in the primary tumor size of metastatic ccRCC, the relationship between ctDNA and tumor size may be not independently present in pT1b-3a ccRCC.

LVI tends to be observed in ccRCC with primary metastatic disease, high Fuhrman grade, and sarcomatoid dedifferentiation, which contributes to the shorter survival time of ccRCC patients.[36,37] FTI and VTT can adversely affect recurrence and survival, particularly when both are present simultaneously.[38] Although the ability of ctDNA analysis to predict the presence of LVI, FTI, and

VTT in non-metastatic ccRCC was not proven in the current study due to an insufficient number of patients, we found that these poor prognostic features tended to be more observed when ctDNA was detected.

In RCC, a higher tumor grade generally denotes increased metastatic probability and decreased overall survival.[12] In non-metastatic ccRCC, tumor grade is considered to be an important factor that predicts poor outcome. However, no relationship between ctDNA and tumor grade was observed in non-metastatic ccRCC in the current study. The low ctDNA detection rate could mask the potential relationship between ctDNA and tumor grade. ctDNA was more frequently observed in pT1b-3a ccRCC, although not statistically significant. ctDNA was frequently detected in metastatic ccRCC, and all metastatic ccRCC were characterized as grade III/IV.

A long-term prospective study is required to determine the clinical significance of ctDNA in non-metastatic ccRCC. Considering that in patients with pT1a non-metastatic RCC, the 5-year cancer-specific survival rate and the 5-year recurrence-free survival rate were 97-100% and 88-96%, respectively, the clinical outcome could not be evaluated in the current study due to the short follow-up period (less than 2 years after surgery).[60,61]

Due to the short follow-up period, we could not correlate clinical outcomes in non-metastatic patients with ctDNA detected. However, we introduced an example case of recurrence after partial nephrectomy for small-sized non-metastatic ccRCC with ctDNA (Patient ID 121). Somatic variants of *VHL* and *NF1* were detected in a pre-operative blood sample from the patient. *VHL* is the most frequently mutated gene, also known as an early driver gene of ccRCC, and variants of *NF1* can be widely found in multiple cancers.[28,50] Somatic variants of CH were not filtered, however, *VHL* and *NF1* genes are rarely detected in CH.[62-64] Although the renal mass was classified as pT1a ccRCC, the WHO/ISUP grade was IV with sarcomatoid differentiation, which is an aggressive feature of ccRCC.[58] This case shows that ctDNA analysis in non-metastatic ccRCC can help

to predict the prognosis and can be a biomarker that aids medical decision making for small renal masses.

Several concerns should be addressed about the feasibility of ctDNA analysis in non-metastatic ccRCC. First, poor reproducibility is expected because the VAF of most somatic variants in ctDNA was less than 0.5% in non-metastatic ccRCC. High reproducibility of ctDNA analysis is typically guaranteed when detecting somatic variants with a VAF higher than 0.5%. However, reproducibility is generally poor when detecting somatic variants with a VAF lower than 0.5%, which remains a challenge in early cancer.[65]

Second, the positive concordance rate of somatic variants between plasma and matched tissue was zero in non-metastatic ccRCC. Although only five matched tissue samples were used to identify somatic variants concordant with ctDNA in non-metastatic ccRCC, three of the five matched tissue samples showed somatic variants of *VHL*, which is the most frequently mutated gene in ccRCC with the potential to be detected in plasma.[28] It is possible that the discordant somatic variants detected in plasma were derived from the parts of ccRCC where the biopsy was not performed. However, the majority of ccRCC show monoclonality of *VHL* rather than multiple clones in early stage.[50] Meanwhile, two matched tissue samples of metastatic ccRCC showed the same somatic variants observed on ctDNA analysis. Considering that high intra-tumoral heterogeneity is more frequently detected in late-stage ccRCC, detecting ctDNA variants that were not observed in the matched tissue samples seemed to complement the overall mutational profiles.[50] A study that included ten localized ccRCC samples with tumor sizes greater than those of the present study showed a similar concordance pattern between ctDNA and matched tissue. In a previous study, somatic variants of *VHL* were detected in more than half of the matched tissue samples, however, only one third of the concordant somatic variants of *VHL* were detected in plasma.[21] Taken overall, ctDNA analysis did not fully represent the characteristics of tumors in non-metastatic ccRCC.

Third, one patient with a large pseudocyst showed ctDNA with a variant of uncertain significance in *MET*. Potential false positives should be interpreted with caution in ctDNA analysis. Although ctDNA from benign tumors is unlikely to be detected by ctDNA analysis, cfDNA can be release in patients with benign disease or inflammatory disease, resulting in overdiagnosis.[49,66] Because the sample size of the benign control group was too small to evaluate the specificity of ctDNA, further studies are needed to demonstrate the effects of benign renal tumors on ctDNA analysis.

In the current study, the possibility that ctDNA variants were CH-derived somatic variants cannot be ruled out. CH is associated with increased risk of hematologic malignancy, cardiovascular disease, and overall mortality, but it can also act as background noise in ctDNA analysis.[18,62] Importantly, CH generally increases with aging, especially from the 60s, onwards.[62] Although matched peripheral blood mononuclear cells were not sequenced to confirm CH somatic variants, and major CH-related genes such as *DNMT3A*, *TET2,* and *ASXL1* were not included in the targeted NGS panel in the present study, the majority of non-metastatic ccRCC patients in the ctDNA detection group were over 60 years old, which suggests that possible somatic variants of CH may have existed. Furthermore, *TP53* and *KDM6A* are related to CH, and in this study, these were the most common mutated genes in non-metastatic ccRCC.[62,63] Several RCC studies report that somatic variants in the range of 9-31% were identified as CH-derived somatic variants.[53,67] Although the prevalence of CH-derived *TP53* somatic variants was confirmed to be very low in the current study, interpretation of somatic variants of *TP53* should be performed cautiously since it is a commonly mutated gene observed in most cancers along with CH.[53,67] Considering the possible CH-derived somatic variants in plasma, the detection rate of ctDNA may decrease and false-positive results may confound the interpretation of ctDNA analysis. Although CH-derived somatic variants were not

filtered out, their possible effects on ctDNA analysis could be emphasized through the present study, especially in non-metastatic ccRCC.

This study has several other limitations. First, the characteristics of ctDNA in non-metastatic ccRCC could not be completely elucidated. Although the sample size of non-metastatic ccRCC patients was greater than previously reported in the literature, the number of somatic variants detected in plasma was too low to fully reveal the characteristics of ctDNA. We learned that it is necessary to increase the sensitivity of ctDNA analysis before exploring the characteristics of ctDNA in ccRCC. In future, strategies should be applied to improve the sensitivity of ctDNA analysis such as ultra-deep sequencing, size selection of cfDNA fragments, and combining other types of genetic alterations (e.g. copy number variation, methylation).[47] Second, the sample size was too small to completely characterize the somatic variants of ctDNA in pT1b-3a non-metastatic ccRCC and metastatic ccRCC. The statistical power was weak when comparing the characteristics of ctDNA between groups. Third, ideally, an accurate concordance rate between plasma and matched tissue samples can be achieved when the biopsy fully reflects the genetic characteristics of the renal tumor. However, since the biopsy was not always performed on multiple sites of a renal tumor, some somatic variants in matched tissue may have been missed. Moreover, somatic variants of matched tissue were compared in selected patients only. These factors could have affected the concordance of somatic variants between plasma and matched tissue samples.

V. CONCLUSION

In this study, the characteristics of ctDNA analysis in non-metastatic ccRCC were explored, with particular focus on pT1a stage disease. ctDNA was analyzed in association with clinicopathological data. Non-metastatic ccRCC was characterized by a low detection rate, low VAF, and a different proportion of genes with ctDNA, compared with metastatic ccRCC. ctDNA was associated

with tumor size and patient age in non-metastatic ccRCC. However, the relationship between ctDNA and tumor grade was not clear. Future prospective studies are required to demonstrate the clinical significance of ctDNA of non-metastatic ccRCC. Improvement of the sensitivity of ctDNA analysis and somatic variants of CH filtration may clarify the characteristics of ctDNA in non-metastatic ccRCC and increase the clinical utility of ctDNA analysis.

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ABSTRACT (IN KOREAN)

비전이성 투명세포 신세포암에서 순환종양 DNA 의 특성

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연구배경: 투명세포 신세포암 (clear cell renal cell carcinoma; ccRCC) 은 신세포암에서 가장 많이 발견되는 유형이다. 순환종양 DNA (circulating-tumor DNA; ctDNA) 는 신장 종괴 생검을 대체하거나 보완할 수 있는 바이오마커 중 하나로 대두되고 있다. 그러나 비전이성 ccRCC 에서, 특히 작은 사이즈의 암종에서의, ctDNA 의 특성은 아직 밝혀지지 않았다.

연구방법: 신장암이 의심되는 종괴에 대하여 외과적 절제를 받은 120명의 환자로부터 수술 전에 혈장을 수집하였다. 그 중 ccRCC 환자의 혈장으로부터 추출한 DNA 를 차세대 시퀀싱 (Next-generation sequencing) 기술을 사용하여 시퀀싱 하였으며 Pi-Seq 알고리즘 (Dxome, 성남, 대한민국) 을 사용하여 ctDNA 를 분석하였다. 비전이성 ccRCC 의 ctDNA 의 특성은 전이성 ccRCC 와 비교되었다. 비전이성 ccRCC 를 pT1a 군과 pT1b-3a 군으로 나누어 ctDNA 와 연관된 조사를 추가적으로 실시하였다. 검출률, 변이의 대립유전자 빈도 및 ctDNA 가 검출된 유전자의 빈도가 평가되었다. ctDNA 는 여러 임상병리학적 특징과 연관되어 조사되었다. 혈장과 매칭된 조직

사이의 체세포 변이의 양성 일치성을 평가하였다.

연구결과: 120명의 환자 중 90명은 ccRCC, 20명은 non-ccRCC 그리고 10명은 양성 종양 환자였다. ccRCC 환자 중 15명의 환자는 특정 과거력으로 인해 연구에서 제외되었다. 75명의 ccRCC 환자 중 67명이 비전이성 환자로 확인되었다. 비전이성 ccRCC 의 대부분은 병리학적 T 단계 1a로 분류되었다 (79.1%). ctDNA 의 검출률은 비전이성과 전이성 ccRCC 에서 각각 26.9%와 75.0% 였으며, 비전이성 ccRCC 에서는 종양의 크기가 커질수록 ctDNA 검출률이 증가하는 경향을 보였다. pT1a ccRCC 의 경우 ctDNA 검출률이 22.6% 였다. 변이 대립유전자 빈도의 중간값은 비전이성과 전이성 ccRCC 에서 각각 0.351% 와 1.168% 였다. 비전이성 ccRCC 에서 ctDNA 가 검출된 유전자의 빈도는 *VHL*, *PBRM1*, *SETD2* 그리고 *BAP1* 이 주로 관찰되는 전이성 ccRCC 와는 차이가 있었다. *NF1*, *TP53* 그리고 *KDM6A* 가 비전이성 ccRCC 에서 자주 관찰되었다. 비전이성 ccRCC 에서 ctDNA 검출은 종양의 크기와 환자의 나이와 연관되었으며 종양의 등급과는 관련이 없었다. ctDNA 는 림프혈관계 침범, 지방조직침범 그리고 정맥종양혈전이 발견되는 경우에서 더 자주 관찰되었으나 통계학적인 차이는 없었다. 비전이성 ccRCC 에서 ctDNA 와 매칭된 조직 간의 양성 일치성은 좋지 않았다.

결론: 비전이성 ccRCC, 특히 pT1a 에 해당하는 작은 크기의 ccRCC 에 대하여 ctDNA 의 특성을 탐색하였다. 낮은 검출률, 변이의 낮은 대립유전자 빈도 그리고 전이성 ccRCC 와 다른 ctDNA 유전자 비율이 나타났다. 비전이성 암에서 ctDNA 는 종양의 크기 및 환자 나이와 연관되었으나 종양의 등급과는 그 관계가 불명확하였다. 여과되지 않은 클론 조혈 가능성이 있는 변이는 분석에서 고려되지 않았으며, 이는 이 연구의 한계 중 하나로 생각된다. 비전이성 ccRCC 에서

ctDNA 의 임상적 유용성을 입증하기 위해 전향적 연구가 실시되어야 한다. 민감도 개선 및 클론 조혈 변이 배제는 ctDNA 분석의 임상적 유용성을 향상시킬 것으로 사료된다.

핵심되는 말 : 순환종양 DNA, 투명 세포 신세포암, 비전이성 암, 작은 신장 종괴