

ctDNA predicts clinical T1a to pathological T3a upstaging after partial nephrectomy

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Abstract

Most patients diagnosed with clear cell renal cell carcinoma (ccRCC) are also detected with small and organ-confined tumors, and the majority of these are classified as clinical tumor stage 1a (cT1a). A considerable proportion of patients with cT1 RCC shows tumor upstaging to pathological stage 3a (pT3a), and these patients have worse oncological outcomes. The role of circulating tumor DNA (ctDNA) in RCC has been limited to monitoring treatment response and resistance. Therefore, the present study aimed to evaluate the potential of ctDNA in predicting pT3a upstaging in cT1a ccRCC. We sequenced plasma samples preoperatively collected from 48 patients who had undergone partial nephrectomy for cT1a ccRCC using data from a prospective cohort RCC. The ctDNA were profiled and compared with clinicopathological ccRCC features to predict pT3a upstaging. Associations between ctDNA, tumor complexity, and pT3a upstaging were evaluated. Tumor complexity was assessed using the anatomical classification system. Univariate analysis used chi-squared and Student's *t*-tests; multivariate analysis considered significant factors from univariate analyses. Of the 48 patients with cT1a ccRCC, 12 (25%) were upstaged to pT3a, with ctDNA detected in 10 (20.8%), predominantly in patients with renal sinus fat invasion (SFI; *n* = 8). Among the pT3a group, ctDNA was detected in 75%, contrasting with only 2.8% in patients with pT1a (1/36). Detection of ctDNA was the only significant preoperative predictor of pT3a upstaging, especially in SFI. This study is the first to suggest ctDNA as

Abbreviations: cc, clear cell; cT1a, clinical stage T1a; ctDNA, circulating tumor DNA; EAU, European Association of Urology; PFI, perinephric fat invasion; PN, partial nephrectomy; pT3a, pathological stage T3a; RCC, renal cell carcinoma; RN, radical nephrectomy; SFI, sinus fat invasion; TE, tumor enucleation.

Jee Soo Park and Hongkyung Kim contributed equally to this work as first authors.

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a preoperative predictor of pT3a RCC upstaging from cT1a based on preoperative radiological images.

KEYWORDS

nephrectomy, pathological stage T3a, perinephric fat invasion, renal cell carcinoma, sinus fat invasion

1 | INTRODUCTION

As imaging examinations have advanced dramatically in recent years,¹ most patients diagnosed with ccRCC are incidentally identified as having small and organ-confined tumors.² The vast majority of these newly diagnosed cases are classified as cT1a (≤ 4 cm).³ Although PN was initially indicated for cT1a masses according to the EAU guidelines,⁴ the indication of PN has expanded to complex and larger renal masses with the adoption of robotic technology,⁵ with data suggesting similar oncological outcomes to those of RN and renal functional benefit.⁶

Among patients with cT1 ccRCC, a non-negligible proportion have tumor upstaging to pT3a at final pathology, ranging from 4% to 9% of all cT1 tumors.^{7,8} Although the prognosis of upstaging cT1–2 to pT3 seems controversial depending on the urological literature,^{9–12} patients with cT1 RCC with pT3a upstaging have worse oncological outcomes.^{13,14} Therefore, accurate prediction of clinically localized renal tumors for pathological upstaging to pT3a is an important issue in PN planning.¹³ Although several studies have analyzed the preoperative risk factors for upstaging to pT3a, which mainly comprises age and tumor size, location, and complexity,^{5,13,15} none of these are used in clinical practice. In addition, although several studies have reported an association between high tumor complexity and pT3a upstaging rates, nephrometry systems have limitations in predicting pT3a upstaging.¹³

Furthermore, among pT3a RCC, renal SFI has a significantly worse prognosis and more aggressive characteristics than PFI,^{9,16–18} as stated in the EAU guidelines.¹⁹ However, previous studies have identified preoperative predictors of overall pT3a with SFI and PFI but did not consider the differences between them.^{5,20}

The clinical application of ctDNA analysis in RCC, including localized small ccRCC, has been investigated,^{21–24} but the role of ctDNA in cT1a is reportedly limited. However, during our prospective cohort study of ctDNA in RCC, we found that its role was limited to monitoring treatment response and resistance, which was based on the primary objective of a previous study.²⁵ Therefore, we attempted to identify and evaluate the role of ctDNA in predicting clinical T1a to pT3a tumor upstaging in comparison with previously established preoperative risk factors.

2 | MATERIALS AND METHODS

2.1 | Study cohort

We queried a prospective cohort database of a Korean ctDNA urologic registry ([ClinicalTrials.gov](https://clinicaltrials.gov) identifier: NCT04197414) between December 2019 and November 2020 that included patients with cT1a ccRCC (≤ 4 cm) undergoing PN. Patients with N1 or M1 disease were excluded. Among those patients, we included all samples that could be analyzed for ctDNA to minimize selection bias. All procedures were performed by urologic oncology surgeons, and the type of operative approach (open vs. laparoscopic/robotic) was determined by the individual attending surgeons. All participants provided informed consent before inclusion in the study. The study was approved by the Institutional Review Board of the Yonsei University Health System (approval no: 4-2019-1039). All methods were performed in accordance with relevant guidelines and regulations.

2.2 | Data collection

Preoperative features of the patients, including age, sex, and clinical tumor size and stage, were collected. Clinical staging was defined according to the eighth edition of the *Cancer Staging Manual* of the American Joint Committee on Cancer.²⁶ All patients were scored according to the Preoperative Aspects and Dimensions Used for an Anatomical (PADUA) classification of renal tumors.²⁷ Clinical staging and PADUA scores were evaluated by two independent urologists and radiologists. Renal masses with a PADUA score ≥ 10 were classified as high-complexity masses. Pathological findings included nucleolar grading according to the most recent International Society of Urological Pathology (ISUP) grading recommendation,²⁸ tumor necrosis, sarcomatoid differentiation, margin status, and stage. The reasons for upstaging were recorded, if present. Survival outcomes included the length of follow-up, as well as overall, cancer-specific, and recurrence-free survival, cause of mortality, and location of and time to recurrence. Recurrence was defined as new lesions demonstrated by follow-up imaging and/or the were compared with demipresence of pathological specimens.

2.3 | Data analysis

The patients were stratified into two cohorts based on the presence of pT3a upstaging (upstaged vs. non-upstaged). These two groups were compared to determine differences in preoperative factors previously identified for predicting pT3a upstaging.^{5,13,15} The groups were specifically compared for differences in terms of patient age, sex, tumor size, PADUA nephrometry complexity, and presence of ctDNA. Clinicopathological parameters, including ISUP grade, tumor necrosis, sarcomatoid differentiation, surgical approach, and length of follow-up, were also compared between the pT3a upstaged and non-upstaged groups.

2.4 | Processing of blood samples

Peripheral blood was collected immediately before the start of surgery. Blood samples were aliquoted into ethylenediaminetetraacetic acid-containing tubes, centrifuged at 1600g for 10 min at 4°C, and then transferred to fresh tubes. The samples were further centrifuged at 4000g for 10 min at 4°C. Plasma samples were stored at -80°C until ctDNA analysis.

2.5 | Library preparation, target capture, and sequencing for plasma samples

Circulating cell-free DNA (cfDNA) was extracted from 3 to 4 mL plasma samples using a Magnetic Serum/Plasma Circulating DNA Kit (Dxome, Sungnam, Republic of Korea). Library preparation was performed using the DxSeq Library prep reagent (Dxome). Sequencing libraries were hybridized with customized probes targeting 16 RCC-related genes previously developed by our team.²⁹ The enriched DNA was amplified, and clusters were generated and sequenced on a NovaSeq 6000 System (Illumina) with 2 × 151 bp reads, targeting an average sequencing depth of 35,000× for each sample. All procedures were performed according to the manufacturer's instructions.

2.6 | Sequencing data analysis

The position indexing sequencing algorithm (PiSeq; Dxome) was used to identify ctDNA mutations. PiSeq is designed to accurately detect mutations with low variant allele frequencies using the positional information of aligned uncollapsed reads generated from conventional library preparation of next-generation sequencing (NGS).³⁰ All mutations were manually inspected using the Integrative Genomic Viewer.³¹

2.7 | Statistical analysis

Characteristics of the participants were reported as means (standard deviation [SD]) for continuous variables and as numbers (%)

for categorical variables. For univariate analysis, chi-squared and Student's *t*-tests were performed for categorical and continuous variables, respectively. Multivariate logistic regression analyses were performed for factors that were significantly associated with the univariate analyses. All statistical analyses were conducted using R 4.0.3 or SPSS software version 23.0 (IBM Corp., Armonk, NY, USA). All tests were two-tailed, and statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Study population

The cohort consisted of 48 patients (mean age, 54.4 years) who underwent PN for a solitary cT1a renal mass (Table 1). Preoperatively, the patients had a mean tumor size of 2.4 cm and the mean length of follow-up was 31.8 months. At the final pathological evaluation, 25% (12 patients) were upstaged to pT3a (Table 2). Upstaging was most frequently noted due to SFI, followed by PFI, whereas upstaging due to collecting system invasion was not observed (Table 2).

During the mean follow-up period of 31.6 months, only one patient experienced recurrence. Although the follow-up period was significantly shorter in the pT3a upstaged group than in the non-upstaged group, recurrence only occurred in the pT3a upstaged group. This patient had a 3.8 cm ISUP grade 4 ccRCC that recurred 7 months postoperatively in the Gerota's fascia with the detection of a Von Hippel-Lindau (VHL) mutation in their ctDNA. No cancer-specific death occurred in either the pT3a upstaged or non-upstaged

TABLE 1 Characteristics of the study population.

Study population (n = 48)	
Age, years	54.4 (11.7 SD)
Sex, n	
Male (%)	34 (70.8%)
Female (%)	14 (29.2%)
Tumor size, cm	2.4 (0.9 SD)
ISUP grade, n (%)	
Low grade (I/II)	32 (66.7%)
High grade (III/IV)	16 (33.3%)
Necrosis, n (%)	2 (4.2%)
Sarcomatoid differentiation, n (%)	1 (2.1%)
Tumor complexity, high (%)	9 (18.8%)
Surgical approach (%)	
Open	17 (35.4%)
(Robot-assisted) Laparoscopic	31 (64.6%)
ctDNA detection, positive (%)	10 (20.8%)
Length of follow-up, months	31.8 (7.0 SD)

Abbreviations: ctDNA, circulating tumor DNA; ISUP, International Society of Urological Pathology; SD, standard deviation.

TABLE 2 Characteristics of clinical T1a tumors upstaged to pathological T3a.

No. of patients	12
Reason for upstaging (%)	
Renal sinus fat invasion	9 (75.0%)
Perirenal fat invasion	2 (16.7%)
Renal or segmental vein invasion	1 (8.3%)
Collecting system invasion	0 (0.0%)
Tumor size, cm	3.0 (0.9 SD)
ISUP grade, n (%)	
Low grade (I/II)	8 (66.7%)
High grade (III/IV)	4 (33.3%)
No. positive surgical margin (%)	0 (0.0%)

Abbreviations: ISUP, International Society of Urological Pathology; SD, standard deviation.

groups. We identified the risk factors associated with pT3a upstaging from cT1a (Table 3).

Multivariate logistic regression analysis was performed to determine factors independently associated with pT3a upstaging (Table 4).

To avoid collinearity of the nephrometry score with tumor size and complexity determined by the PADUA score, we performed two separate analyses. The first model included patient age, tumor size, and ctDNA presence, whereas the second model included age, tumor complexity, and ctDNA presence. In the first model, tumor size and ctDNA presence were independently associated with pT3a upstaging. In the second model, tumor complexity and the presence of ctDNA were independently associated with this outcome.

ctDNA was detected in 10 of 48 patients (detection rate, 20.8%), who only had one type of ctDNA present (Figure 1).

TABLE 3 Preoperative variables associated with clinical T1a to pathological T3a upstaging.

	Non-upstaged	Upstaged to T3a	p-value
No. of patients	36	12	
Age, years	52.3 (11.9 SD)	60.9 (8.5 SD)	0.025
Sex, n			0.726
Male (%)	26 (72.2%)	8 (66.7%)	
Female (%)	10 (27.8%)	4 (33.3%)	
Tumor size, cm	2.2 (0.8 SD)	3.0 (0.9 SD)	0.007
ISUP grade, n (%)			0.829
Low grade (I/II)	24 (66.7%)	8 (66.7%)	
High grade (III/IV)	12 (33.3%)	4 (33.3%)	
Necrosis, n (%)	2 (5.6%)	0 (0.0%)	0.404
Sarcomatoid differentiation, n (%)	0 (0.0%)	1 (8.3%)	0.08
Tumor complexity, high (%)	1 (2.8%)	8 (66.7%)	<0.001
Surgical approach (%)			0.601
Open	12 (33.3%)	5 (41.7%)	
(Robot-assisted) Laparoscopic	24 (66.7%)	7 (58.3%)	
ctDNA detection, positive (%)	1 (2.8%)	9 (75.0%)	<0.001
Length of follow-up, months	33.5 (4.4 SD)	26.4 (10.4 SD)	0.026

Abbreviations: ctDNA, circulating tumor DNA; ISUP, International Society of Urological Pathology; SD, standard deviation.

TABLE 4 Multivariate analysis of variables associated with clinical T1a to pathological T3a.

Covariate	Model 1 ^a		Model 2 ^b	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Age	1.05 (0.94–1.18)	0.391	1.15 (0.99–1.34)	0.077
Tumor size	12.15 (1.14–129.71)	0.039		
Tumor complexity, high			75.06 (1.82–3095.73)	0.023
ctDNA detection, positive	626.88 (7.31–53749.30)	0.005	50.19 (1.96–1282.81)	0.018

Abbreviations: CI, confidence interval; ctDNA, circulating tumor DNA; OR, odds ratio.

^aModel 1: adjusted for age, tumor size, and the presence of ctDNA.

^bModel 2: adjusted for age, tumor complexity (high), and the presence of ctDNA.

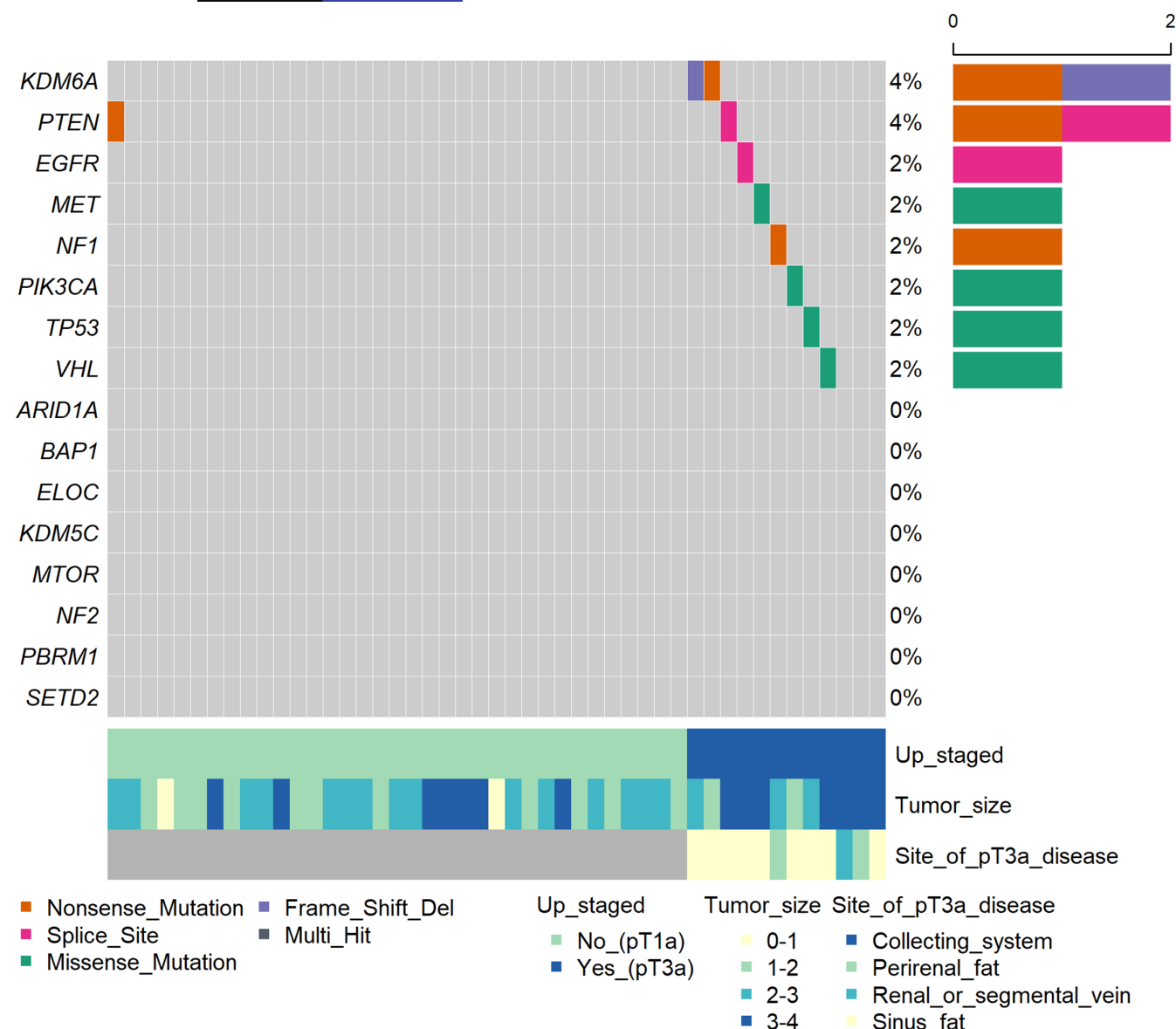


FIGURE 1 ctDNA genomic profile in 48 patients with clinical stage T1a clear cell renal cell carcinoma. The top heatmap shows ctDNA detection events, with their percentages among the total number of patients on the right. The right histogram shows the number of ctDNAs per gene. The bottom heatmap shows information about the upstaged information (including the site of pT3a disease), tumor size, and grade per sample.

KDM5A and PTEN were detected in two ctDNAs, whereas only one ctDNA was found in EGFR, MET, NF1, PIK3CA, TP53, and VHL. Patients with ctDNA in KDM6A and nonsense mutations were not upstaged to pT3a. Among nine of the patients with ctDNA, eight were upstaged to pT3a because of SFI, whereas one was upstaged because of PFI. Patients upstaged because of PFI harbored a nonsense mutation in NF1. The most common mutations were splice-site and missense mutations.

As most ctDNAs were detected in patients upstaged to pT3a with SFI, we performed multivariate logistic regression analysis to determine the factors independently associated with this outcome (Table 5). Both models showed that ctDNA presence was the only statistically significant factor associated with pT3a upstaging due to SFI.

4 | DISCUSSION

Here, almost 25% of all patients treated with PN for cT1a renal masses were upstaged to pT3a ccRCC. Among these cases, ctDNA was detected in 75%, and ctDNA detection was the most significant preoperative predictor of cT1a upstaging to pT3a, especially with SFI. Although preoperative factors that stratify cT1 and cT3a have been previously investigated, no definite parameters that accurately predict pT3a upstaging exist. As there remain concerns about whether patients with pT3a ccRCC who underwent PN would have been better served by RN,⁵ preoperative prediction of pT3a upstaging would be helpful for clinicians to decide between these treatment methods.

TABLE 5 Multivariate analysis of variables associated with clinical T1a to pathological T3a upstaging with renal SFI.

Covariate	Model 1 ^a		Model 2 ^b	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Age	1.04 (0.91–1.20)	0.563	1.10 (0.92–1.31)	0.29
Tumor size	2.89 (0.49–17.14)	0.243		
Tumor complexity, high			28.38 (0.96–842.01)	0.053
ctDNA detection, positive	174.07 (9.28–3264.67)	<0.001	41.29 (2.37–718.70)	0.011

Abbreviations: CI, confidence interval; ctDNA, circulating tumor DNA; SFI, sinus fat invasion; OR, odds ratio.

^aModel 1: adjusted for age, tumor size, and the presence of ctDNA.

^bModel 2: adjusted for age, tumor complexity (high), and the presence of ctDNA.

Although T3a fat invasion in RCC is considered rare,¹⁸ two recent meta-analyses reported that ~6% of cases are upstaged to pT3a from cT1.^{14,15} Furthermore, the incidence of upstaging was highly variable, with percentages increasing to 31%,³² which is similar to the 25% reported in our study. Considering that pT3a upstaging has a poor prognosis,^{13,14} the importance of predicting upstaging to pT3a should again be highlighted. Furthermore, because SFI has a significantly worse prognosis than PFI,^{9,16–18} research on the prediction of pT3a using SFI is urgently needed.

Classification of renal capsular invasion patterns was once considered an unreliable prognostic factor because there was no standardized pathological protocol for capsular invasion in the early years.³³ The situation is similar for fat invasion, where histopathological examination of perinephric fat is not routinely conducted and neither is renal sinus fat systematically collected during PN.³⁴ The unestablished protocol for fat invasion has been further aggravated by the enthusiasm in PN, where it has been further expanded to TE due to advancements in robotic technologies.³⁵ These trends may have led to an underestimation of the incidences of SFI and PFI.¹⁸ Although PN and TE are beneficial for restoring kidney function, a standardized protocol for evaluating fat invasion is necessary, especially for cT1a renal masses.

T3a RCC includes two types of fat invasion: PFI and SFI.¹⁸ Although previous studies have focused on comparing the differences in prognosis between SFI and PFI, where SFI was concluded to have a worse prognosis than PFI,¹⁸ no studies have investigated the predictors of pT3a with SFI. SFI is expected to have worse oncological outcomes than PFI because of the presence of abundant veins and lymphatics in the sinus.³⁶ SFI is the principal route for the extrarenal extension of RCC and is a prognostic factor for poor recurrence-free and overall survival after nephrectomy.^{16,37} These findings are consistent with our result showing that the ctDNA detection rate was higher in patients with SFI.

Here, tumor complexity measured using the PADUA system was significantly associated with the prediction of upstaging to pT3a; however, it was not a significant parameter for predicting upstaging to pT3a with SFI. Higher tumor complexity is associated with higher upstaging rates from cT1 to pT3a after PN.^{5,32} Although the PADUA system can predict fat invasion to some extent, it is not sufficient to predict SFI or PFI.³⁷ As this system was originally designed

for evaluating the surgical complexity and morbidity of PN,¹³ the nephrometry system may be insufficient to predict SFI because tumor building into the renal sinus may not necessarily be a result of SFI.³⁷

The role of ctDNA has primarily been investigated in the monitoring of tumor progression during treatment because of the advantages of non-invasive ctDNA sampling methods.²⁴ Therefore, most of these studies were conducted on metastatic or large-sized ccRCCs.²⁴ Eight studies applied targeted panel sequencing of plasma DNA, the same method used here,²⁴ and these studies were primarily performed in patients with metastatic disease. The number of studies evaluating ctDNA in small-sized non-metastatic RCCs is small due to the low ctDNA detection rates in small RCCs.²⁴ Only two studies have evaluated ctDNA in non-metastatic RCC, with detection rates of 30 and 19%,^{21,22} which is similar to the 20.8% detection rate observed in our study.

The ctDNA detection rates and number of mutations in ctDNAs are significantly higher in tumors with a higher tumor burden.³⁸ Therefore, the use of ctDNA in cT1a ccRCC has not been widely accepted. However, the ctDNA detection rate in cT1a upstaged to pT3a with SFI was 88.9%. Such a high ctDNA detection rate might be because our study used panels with only 16 genes compared with other studies on metastatic RCC with ctDNA detection rates of 19 and 28% that used 10 or 27 genes, respectively.^{21,39} The strong correlation between upstaged pT3a with SFI and ctDNA suggests the role of ctDNA as a significant predictor of this type of upstaging.

In the multivariate analysis, age was not a significant predictor of pT3a upstaging. Vecchia et al. found an association between age and upstaging in a meta-analysis of 13 studies,¹⁵ which might be due to the known biology of RCC, where increased age causes the down-regulation of genes involved in the extracellular matrix in tumors, deregulation of collagens, and increased karyotype mutations.¹⁵ However, Gorin et al. found no association between age and upstaging.⁵ Age was likely to be not a significant predictor of pT3 upstaging because of the inclusion of only small masses that are usually detected in the early years by routine health examinations.

Our study had limitations. First, due to the study design, blood samples were only collected before surgeries. Although checking the mutation status before and after surgeries would provide more accurate information, this study was not aimed to provide the exact

ctDNA mutation status of the study population, but to suggest the use of ctDNA in predicting pT3a upstaging. However, future studies will incorporate blood samples after the surgeries by modifying the study design. Second, the relatively small sample size from one institution undoubtedly limited our ability to discern differences between patients upstaged to pT3a and those not upstaged. Third, we included only ccRCC categories and not non-clear cell categories, which may have introduced bias. Fourth, although we tried to minimize selection bias by including all patients in the study period with their blood samples available with no intervention from researchers, selection bias still could occur. Nonetheless, our findings represent the first analysis of the potential of ctDNA as a predictive factor for pT3a upstaging. Given the limitations of our analysis, our findings should be considered hypothesis-forming and warrant further confirmation.

We demonstrated the potential clinical utility of ctDNA in predicting upstaging of cT1a to pT3a with SFI. As upstaging to pT3a could jeopardize cancer control and increase the oncological safety of PN,¹⁵ studies have been performed to improve the understanding of preoperative factors aiding the stratification of patients for PN.⁵ However, these studies did not provide clinically useful parameters for predicting upstaging to pT3a. Therefore, due to the growing incidence of cT1a RCCs and the selection of PN or TE becoming mainstream, the need for an accurate diagnosis between cT1a and cT3a is becoming more important. We suggest that ctDNA may complement preoperative surgical decisions between PN and RN by accurately predicting upstaging to pT3a, especially with SFI.

AUTHOR CONTRIBUTIONS

Jee Soo Park: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; writing – original draft. **Hongkyung Kim:** Conceptualization; data curation; formal analysis; investigation; methodology; software; visualization; writing – review and editing. **Won Sik Jang:** Investigation; resources; writing – review and editing. **Jongchan Kim:** Investigation; resources; writing – review and editing. **Won Sik Ham:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – review and editing. **Seung-Tae Lee:** Data curation; formal analysis; project administration; resources; software; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed in this study are accessible from the corresponding author upon reasonable request.

ETHICS STATEMENT

All participants provided informed consent before inclusion in the study. The study was approved by the Institutional Review Board of the Yonsei University Health System (approval no: 4–2019-1039). Informed Consent: Informed consent was obtained from all participants.

Registry and the Registration No. of the study/trial: [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=NCT04197414) NCT04197414.

Animal Studies: N/A.

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