

Title: Utility of liquid biopsy using urine in patients with pancreatic ductal adenocarcinoma

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Abstract

In recent years, liquid biopsy for blood and body fluid in cancer patients has attracted attention. However, there have been few reports of liquid biopsy focusing on urine of pancreatic ductal adenocarcinoma (PDAC).

In 56 patients with PDAC, DNA was extracted from urine and plasma prior to treatment, and KRAS mutations were analyzed with droplet digital PCR to examine the mutation detection rate. Our study showed that KRAS mutations were found in 27 cases (48%) in urine and 27 cases (48%) in plasma. The detection rate of urine KRAS mutations varied by renal functions. The rates were 70% (14/20) and 36% (13/36) in the creatinine clearance rate (CCr) < 70 mL/min group and in the CCr ≥ 70 mL/min group, respectively (P=0.024). Whereas, no influence of the CCr was observed in the detection rates of plasma KRAS mutations. The rates were 50% (10/20) and 47% (17/36) in cases with the CCr < 70 mL/min group and the CCr ≥ 70 mL/min group, respectively.

Although the sample size was small, this study clearly indicated a new possibility of less invasive urine liquid biopsy in PDAC patient.

Introduction

Liquid biopsy, for detecting molecules such as circulating tumor cells (CTC), circulating cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), and exosomes in peripheral blood and other body fluids from cancer patients, has been developed recently^{1 2 3}. Detecting these molecules enables not only early diagnosis and improved prognosis but also monitoring and prediction of treatment response⁴. A blood sample is widely used for liquid biopsy, whereas other body fluid samples such as saliva, cerebrospinal fluid (CSF), bile, urine, and stools have been reported for liquid biopsy⁴. Of these samples, urine may have a remarkable advantage as an ultra-non-invasive, simple sample source over tissue and blood.

Urinary cell-free DNA (ucfDNA) originates either from the shedding of cells into urine from the genitourinary tract or from cfDNA and ctDNA in circulation passing through glomerular filtration, known as transrenal DNA. Next generation sequencing (NGS) and digital PCR could make it possible to detect rare mutations and methylation for ucfDNA. UcfDNA has been applied for not only urological cancer but also non-urological cancers such as colon cancer, lung cancer, hepatocellular cancer, and

gastric cancer. However, to our knowledge, ucfDNA for pancreatic ductal adenocarcinoma (PDAC) with NGS or digital PCR has not been reported.

The KRAS oncogene is a driver mutation that is presented in greater than 90% of PDAC. The concordance rate between tissue DNA and blood ctDNA with a KRAS mutation was reported to be 60–80%^{5 6}. Detection of a KRAS mutation in blood could be used to improve prognosis and monitor treatment and disease progression. If we could gather the same information from a urinary liquid biopsy, we could have an ideal, non-invasive option for cancer treatment.

In this study, we analyzed KRAS mutations in urine and investigated the potential of urine liquid biopsy in patients with PDAC.

Materials and Methods

Patients

We enrolled 56 patients with PDAC who were admitted to Okayama University Hospital between May 2015 and April 2018. All patients were diagnosed histopathologically. We investigated the diurnal variation of cfDNA concentration in

urine for 9 patients as a preliminary step. Then, we investigated the detection rate of KRAS mutations (G12D, G12V) in urine and plasma for 56 patients. In 40 of the 56 patients, we also examined the KRAS mutations in tissue using a needle washing solution after fine needle aspiration (FNA). All patients provided written informed consent prior to enrolment. This study was approved by the ethics committee of Okayama University Hospital, conducted in accordance with the Declaration of Helsinki.

DNA extraction

All samples were collected prior to initial treatment. DNA was extracted from each source according to the manufacturer's instructions. Urine samples were collected and stored in tubes with ethylenediaminetetraacetic acid (EDTA) at -80°C for later DNA extraction. After removal of the sediment by centrifugation (16000×g, 10 min, room temperature), ucfDNA was extracted from 3 mL of urine with a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). Plasma samples were separated by centrifugation (3000 rpm, 10 min, 4°C) within 3 h of blood collection. The samples were then stored at -30°C for later DNA extraction. CfDNA was extracted from 1 mL of

plasma with the same kit. Needle washing solutions after FNA for diagnosis were stored at -30°C for later DNA extraction. CfDNA was extracted from 200 µL of the solutions by a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). All DNAs were eluted in a final volume of 50 µL and stored at -30°C. DNAs extracted from plasma and urine were quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Droplet digital PCR

The presence of KRAS mutations was detected via droplet digital PCR (QX200 system; Bio-Rad Laboratories, Hercules, CA, USA) using the two probes (G12D and G12V), as follows: DNA eluent (5 µL) from urine, plasma, and needle washing solution was mixed with Droplet PCR Supermix (10 µL; Bio-Rad Laboratories, Hercules, CA, USA), primer/probe mixture (2 µL), and sterile DNase- and RNase-free water (5 µL). The mixture (22 µL) was added to droplet generation oil (70 µL; Bio-Rad Laboratories, Hercules, CA, USA) to produce a droplet. The emulsion was thermal cycled as follows: an initial temperature setting of 95°C for 10 min, 40 cycles at 94°C for 30 s, and 55°C for 1 min. A final step of 98°C for 10 min for enzyme deactivation was performed, and the

reaction mixes were analyzed using a droplet reader (Bio-Rad Laboratories, Hercules, CA, USA). For quantification, a fluorescence signal readout was obtained using QuantaSoft software (Bio-Rad Laboratories, Hercules, CA, USA). In this study, we set the threshold of fluorescence intensity as 2000 using a positive control and a negative control, which were cancerous and non-cancerous lesion tissues of surgical resection samples in patients with PDAC. All experiments were conducted in duplicate to diminish the effect of a false negative result, although its frequency was low in these materials. A positive result was accepted if it was positive even once.

Statistical analysis

We used the software JMP v14.0 (SAS Institute, Cary, NC, USA) and set the significance at $P < 0.05$. The Wilcoxon test (non-parametric) was used for the analysis of continuous data, and the Chi-square test was used for the analysis of categorical data. Survival curves were generated by the Kaplan–Meier method and compared by log rank test.

DNA fragment analysis

We analyzed the DNA fragments of ucfDNA by Qsep100 DNA Fragment Analyzer (BiOptic inc, Taiwan).

Results

Diurnal variation of cell-free DNA concentration in urine

Prior to the study of KRAS mutations in urine, we investigated diurnal variation of ucfDNA concentration because no data about the variation had been reported. We collected urine at 7:00 am, 11:00 am, 3:00 pm, and 7:00 pm from the first 9 PDAC patients, and measured the DNA concentration (Figure 1). Median concentrations were 8.5 ng/mL, 5.6 ng/mL, 11.2 ng/mL, and 14.5 ng/mL, respectively, but the variation was not significant ($P=0.321$). Therefore, we decided not to define the time of collecting urine samples in subsequent analyses.

Patient characteristics

The characteristics of 56 patients at baseline are shown in Table 1. Median patient age was 66.5 years, and 30 patients (54%) were male. Creatinine clearance rate

(CCr) ranged from 23.9 mL/min to 164.5 mL/min, with a median of 74.1 mL/min.

Median tumor size was 35 mm. About half of the cases were stage IV, and none were stage I. Seven patients underwent surgical resection after neo-adjuvant chemotherapy, and 48 patients received chemotherapy (modified FOLFIRINOX for 17 cases, gemcitabine plus nab-paclitaxel for 28 cases, and other drugs for 3 cases). For the stage IV patients, only one patient had renal metastasis.

Detection of KRAS mutations

In urine, G12D and G12V were detected in 25 of 56 patients (45%) and 4 of 56 patients (7%), respectively. The detection rate of KRAS mutations (G12D and/or G12V) in urine was 48% (27 of 56 patients). In plasma, G12D and G12V were detected in 17 of 56 patients (30%) and 11 of 56 patients (20%), respectively. The detection rate of KRAS mutations (G12D and/or G12V) in plasma was 48% (27 of 56 patients), which was equal to that in urine (Table 2).

The median value of cfDNA concentration was 16.5 ng/mL in plasma and 5.7 ng/mL in urine (Table 2). CfDNA concentration in plasma and urine was significantly different ($P < 0.001$).

Effect of renal function

One patient did not receive treatment. Patient age and existence of diabetes mellitus were statistically different between the $CCr < 70$ mL/min group and the $CCr \geq 70$ mL/min group, but no other factor was statistically different between the two groups (Table 3). For stage IV patients, there was a significant difference only in age ($P < 0.001$, median age for the $CCr < 70$ mL/min group and the $CCr \geq 70$ mL/min group of 75 and 58, respectively), but no significant difference was observed in other factors including tumor marker and tumor size.

DNA concentration in plasma and urine, divided into two groups by CCr , is shown in Table 4. In the $CCr < 70$ mL/min group, cfDNA concentration in plasma and urine was not significantly different ($P = 0.098$); however, it was significantly different ($P < 0.001$) in the $CCr \geq 70$ mL/min group. The mean concentration of urinary cfDNA in

the $CCr < 70$ mL/min group was higher than that in the $CCr \geq 70$ mL/min group (Table 4; $P=0.038$). In plasma, there was no influence of CCr for DNA concentration (Table 4; $P=0.993$). These results may suggest that cfDNA tends to translocate the kidney barrier with renal function degeneracy.

Interestingly, the detection rate in urine was remarkably different in the reexamination by renal function, because the detection rate was 70% (14/20) in the $CCr < 70$ mL/min group, in contrast to 36% (13/36) in the $CCr \geq 70$ mL/min group. The detection rate was significantly higher in the group with the lower CCr ($P=0.024$). In plasma, the detection rate was 50% (10/20) in the $CCr < 70$ mL/min group and 47% (17/36) in the $CCr \geq 70$ mL/min group, but the influence of CCr for plasma mutation was not clear ($P=1.00$; Figure 2).

In tissue, G12D and G12V were detected in 28 of 40 patients (70%) and 12 of 40 patients (30%), respectively. The detection rate of KRAS mutations (G12D and/or G12V) in tissue was 83% (33 of 40 patients). The sensitivity of the urine liquid biopsy was 42% (14/33). After separation of the low and high CCr groups, the sensitivity was 80% (8/10 cases) in the $CCr < 70$ mL/min group and 26% (6/23 cases) in the $CCr \geq 70$

mL/min group (Figure 3). The sensitivity was significantly higher in the group with the lower CCr (P=0.007). On the other hand, the sensitivity of plasma liquid biopsy was 70% (7/10 cases) in the CCr < 70 mL/min group and 48% (11/23 cases) in the CCr ≥ 70 mL/min group (Figure 3), but the influence of CCr for sensitivity was not clear (P=0.282). Liquid biopsy on urine showed the influence of renal function on detection rate and sensitivity, unlike plasma.

DNA fragment size

The size of ucfDNA was a bimodal distribution of about 200 bp and 1000bp (Figure 4).

Discussion

The detection of KRAS mutations in blood from PDAC patients is possible, and we have reported its utility for diagnosis, treatment, and prognosis^{5 7}. However, there have been no reports for liquid biopsy using urine in PDAC patients. In this study, we showed that detection of KRAS mutations in urine from PDAC patients was possible, and the rate was comparable to that with plasma. The detection rate of urine KRAS mutations

was high especially in patients with poor renal function and was higher than that of plasma in these patients.

KRAS mutations present in urine from cancer patients were reported to be detectable, and the usefulness of analyzing mutations was reported. In PDAC patients, KRAS mutations have been detected previously by PCR⁸. However, the utility of analyzing these mutations is unknown. Transrenal DNA has been identified, but the mechanism of transfer is unclear. The non-globular shape or the deformability of the DNA complexes may enable them to pass through the glomerular filtration. Another explanation is that cfDNA may be included in liposomes; therefore, the cfDNA could pass through the glomerular filtration^{9 10}.

In this study, we found that ucfDNA concentration varied by renal function, and this fact suggested that the renal barrier becomes more permeable to cfDNA due to renal function degeneracy. An increase of urine protein with renal function degeneracy is well known. It is considered that the size barrier of glomerular filtration collapses with renal function degeneracy because podocytes, which are responsible for the size barrier, tend to disappear¹¹. With renal function degeneracy, the charge barrier function may

decrease too. Therefore, albumin and other proteins pass the barrier. Considering this mechanism, dysfunction of the barrier may affect the diffusibility of cfDNA. The size of cfDNA in blood is reported to be about 180 to 200 bp¹². UcfDNA can be classified generally by its size into high-molecular-weight and low-molecular-weight.

Low-molecular-weight DNA is about 250 bp¹³ or shorter¹⁴, and these DNAs originate either from circulation or apoptotic cells in contact with urine^{13 15 16}. We analyzed the DNA fragments of ucfDNA and the result was about 200 bp. We speculate that high-molecular-weight DNA cannot pass easily through the glomerular filtration barrier, but low-molecular-weight DNA can. Targeting of ucfDNA from circulation passing through the kidney barrier might further increase the detection rate and sensitivity of the urine liquid biopsy.

On the other hand, we tried to address stage specific confounders leading to changes in renal function. However, no significant difference in characteristics but age was observed between the $CCr < 70$ mL/min group and the $CCr \geq 70$ mL/min group.

In non-urological cancers with urine liquid biopsy, mutation rate rather than methylation and integrity was mainly studied. EGFR and KRAS mutations in NSCLC¹⁷

^{18 19 20}, KRAS and TP53 mutations in colorectal cancer ^{21 22 13}, EGFR mutations in gastric cancer ²³, and in other cancers have been reported, but not for PDAC. Chen et al. reported that the concordance rate of EGFR mutations in NSCLC patients between tissue and ucfDNA was 88% ¹⁷. Fajiu et al. reported that the concordance rate of EGFR mutations in NSCLC patients between tissue and ucfDNA was 42% in the early stage, and 93% in the advanced stage ¹⁸. In this study, the concordance rate of KRAS mutations between tissue and ucfDNA was 47.5% (19/40). In 56 patients, seventeen patients with positive KRAS mutation in the plasma also had the same KRAS mutation in the urine. On the other hand, ten patients with KRAS mutation in urine had no KRAS mutation in plasma. The false positive and false negative rate of using this urine testing method were 37% (10/27) and 34.4% (10/29), respectively. Concordance rate between urine KRAS mutation and plasma KRAS mutation was 64.2% (36/56). It is difficult to compare these results because the type of cancer was different, and the results might have been affected by sample volume. We used 1 ml of plasma and 3 ml of urine, but a greater volume (10 ml) was used in the previous studies. In terms of urine liquid biopsy, a larger amount of sample is available, and it is less invasive, which is the greatest advantage. In NSCLC

patients, the sensitivity of EGFR mutations analyzed by next-generation sequencing assays was higher in a larger volume of urine sample ¹⁹. If we had used a larger volume of urine sample, the results might have been even more significant.

Urine liquid biopsy, a less invasive technique, is also feasible. This study suggested the potential of urine liquid biopsy compared with plasma liquid biopsy, but the clinical effect is still unknown. We conducted a sub-analysis about progression free survival (PFS) and overall survival (OS) for patients who received systemic chemotherapy. For plasma liquid biopsy, there was no significant difference in PFS between the group with KRAS mutations and that without mutations (P=0.451, median PFS for KRAS-positive and KRAS-negative patients of 138 and 201 days, respectively). The same result was obtained by urine liquid biopsy (P=0.935, median PFS for KRAS-positive and KRAS-negative patients of 160 and 154 days, respectively). There was no significant difference in OS between the group with KRAS mutations and that without mutations for plasma liquid biopsy (P=0.261, median survival times for KRAS-positive and KRAS-negative patients of 406 and 449 days, respectively) or urine liquid biopsy (P=0.882, median survival times for KRAS-positive and KRAS-negative

patients of 470 and 449 days, respectively). Further study will reveal the clinical effect of urine liquid biopsy in the context of renal function.

The limitations of the current study are shown below. This was a single center study. The patient characteristics were also biased, and there was no early disease stage. Also, the number of patients enrolled in this study was small. Therefore, all stages and a larger number of patients should be enrolled in further studies. Further, we substituted the needle washing solutions after FNA for tissue samples. More than 90% of cases of pancreatic intraepithelial neoplasia of all grades have KRAS mutations²⁴, and the detection rate of FNA samples was 83% in our study. Finally, we analyzed KRAS mutations of G12D and G12V, but other mutations such as G12R, G13D, and Q61L were not investigated. G12D and G12V are the most common KRAS mutations²⁵, but other mutations could have been analyzed.

In conclusion, urine liquid biopsy in PDAC patients is a simple and ultra-non-invasive technique. Moreover, a sufficient amount of urine sample can be easily secured, unlike for other samples. The detection rate and the sensitivity are comparable to plasma liquid biopsy and are higher for PDAC patients with renal function

degeneracy. Further investigation might allow the technique to substitute for liquid biopsy using a blood sample.

Figure legends

Figure 1. Urine at 7:00 am, 11:00 am, 3:00 pm, and 7:00 pm from the first 9 PDAC patients was collected, and the DNA concentration measured. Median concentrations were 8.5 ng/mL, 5.6 ng/mL, 11.2 ng/mL, and 14.5 ng/mL, respectively, but the variation was not significant ($P=0.321$).

Figure 2. In urine, the detection rate of KRAS mutations was 70% (14/20) with the $CCr < 70$ mL/min group, in contrast to 36% (13/36) with the $CCr \geq 70$ mL/min group. The detection rate was significantly higher in the group with the lower CCr ($P=0.024$). In plasma, the detection rate was 50% (10/20) in the $CCr < 70$ mL/min group and 47% (17/36) in the $CCr \geq 70$ mL/min group, but the influence of CCr for plasma mutation was not clear ($P=1.00$).

Figure 3. The sensitivity of the urine liquid biopsy was 42% (14/33). The sensitivity was 80% (8/10 cases) in the $CCr < 70$ mL/min group and 26% (6/23 cases) in the $CCr \geq 70$ mL/min group. The sensitivity was significantly higher in the group with the lower CCr ($P=0.007$). In contrast, the sensitivity of plasma liquid biopsy was 70% (7/10 cases) in the

CCr < 70 mL/min group and 48% (11/23 cases) in the 70 mL/min ≤ CCr group, but the influence of CCr for sensitivity was not clear (P=0.282).

Figure 4. Size distribution of ucfDNA samples from patients with PDAC was analyzed.

All samples showed a bimodal distribution of about 200 bp and 1000bp, indicating that

ucfDNA originates either from cfDNA and ctDNA in circulation passing through

glomerular filtration or from the shedding of cells into urine from the genitourinary tract.

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Table 1. Patient Characteristics

	N = 56
Age, years (range)	66.5 (38–91)
Sex, male (%)	30 (54%)
Diabetes mellitus, no. (%)	19 (34%)
Creatinine, mg/dL (range)	0.6 (0.4–1.4)
CEA, ng/mL (range)	3.9 (0.7–274)
CA19-9, IU/mL (range)	233.9 (0.9–129164)
SPAN1, U/mL (range)	96.8 (10–28000)
DUPAN2, U/mL (range)	348 (25–16000)

Tumor location (head of pancreas), no. (%)		25 (45%)
Tumor size, mm (range)		35 (18–80)
Stage of disease (UICC), no. (%)	I	0 (0%)
	II	12 (21.5%)
	III	18 (32%)
	IV	26 (46.5%)
Initial therapy, no. (%)	Chemotherapy	48 (86%)
	NAC + surgical resection	7 (12%)
	BSC	1 (2%)

CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; NAC: neoadjuvant chemotherapy

Table 2. Comparison between plasma and urine samples in patients with PADC

	Plasma (N = 56)	Urine (N = 56)	P value
Detection rate of KRAS mutation, no. (%)	27 (48%)	27 (48%)	1.000
DNA concentration, ng/mL (IQR)	16.5 (12.5–22.5)	5.7 (3.3–12.4)	<0.001

Table 3. Patient Characteristics by CCr

	CCr < 70 (N = 20)	CCr ≥ 70 (N = 36)	P value
Age, years (range)	75 (63–91)	61.5 (38–71)	<0.001
Sex, male (%)	10 (50%)	20 (56%)	0.782
Diabetes mellitus, no. (%)	3 (15%)	16 (44%)	0.038
CEA, ng/mL (range)	5.0 (1.0–115.8)	3.9 (0.7–274)	0.521
CA19-9, IU/mL (range)	280.5 (0.9–129164)	194.4 (1.7–50600)	0.918
SPAN1, U/mL (range)	84.4 (10–28000)	110.7 (10–11000)	0.681
DUPAN2, U/mL (range)	348 (25–16000)	294 (25–16000)	0.632
Tumor location (head of pancreas), no. (%)	8 (40%)	17 (47%)	0.779

Tumor size, mm (range)		35.5 (20–60)	35 (18–80)	0.803
Stage of disease (UICC), no. (%)	I	0 (0%)	0 (0%)	0.286
	II	3 (15%)	9 (25%)	
	III	6 (30%)	12 (33%)	
	IV	11 (55%)	15 (42%)	
Initial therapy, no. (%)	Chemotherapy	16 (80%)	32 (89%)	0.912
	NAC + surgical resection	3 (15%)	4 (11%)	
	BSC	1 (5%)	0 (0%)	

Table 4. Comparison between CCr < 70 and CCr ≥ 70

	Plasma (N = 56)			Urine (N = 56)		
	CCr < 70	CCr ≥ 70	P value	CCr < 70	CCr ≥ 70	P value
	(N = 20)	(N = 36)		(N = 20)	(N = 36)	
Detection rate of KRAS mutation, no. (%)	10 (50%)	17 (47%)	1.000	14 (70%)	13 (36%)	0.024
DNA concentration, ng/mL (IQR)	16.9 (12.2–24.4)	16.2 (13.0–21.7)	0.993	8.0 (3.5–40.3)	5.5 (2.9–9.1)	0.038

Figure 1

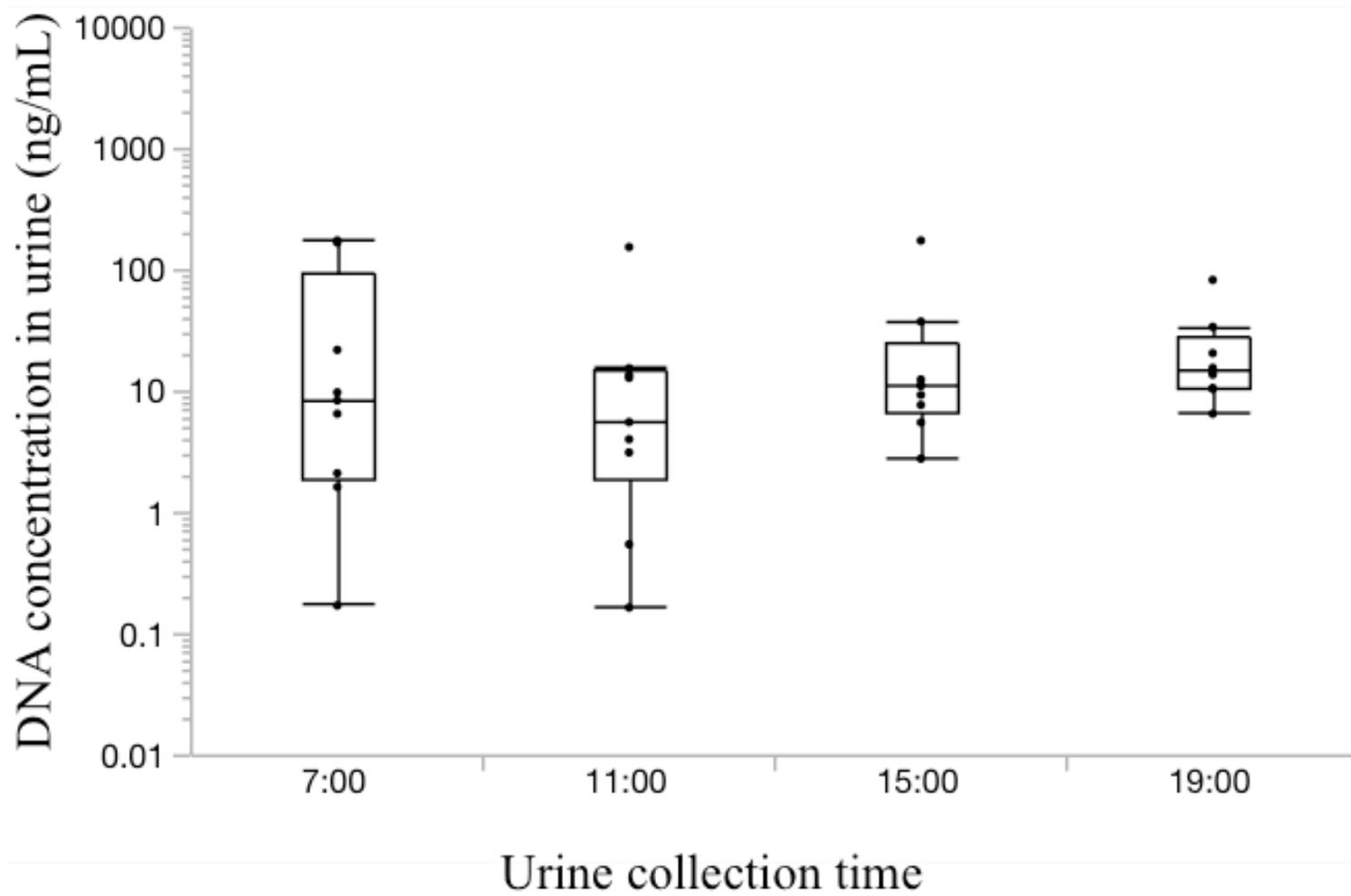


Figure 2

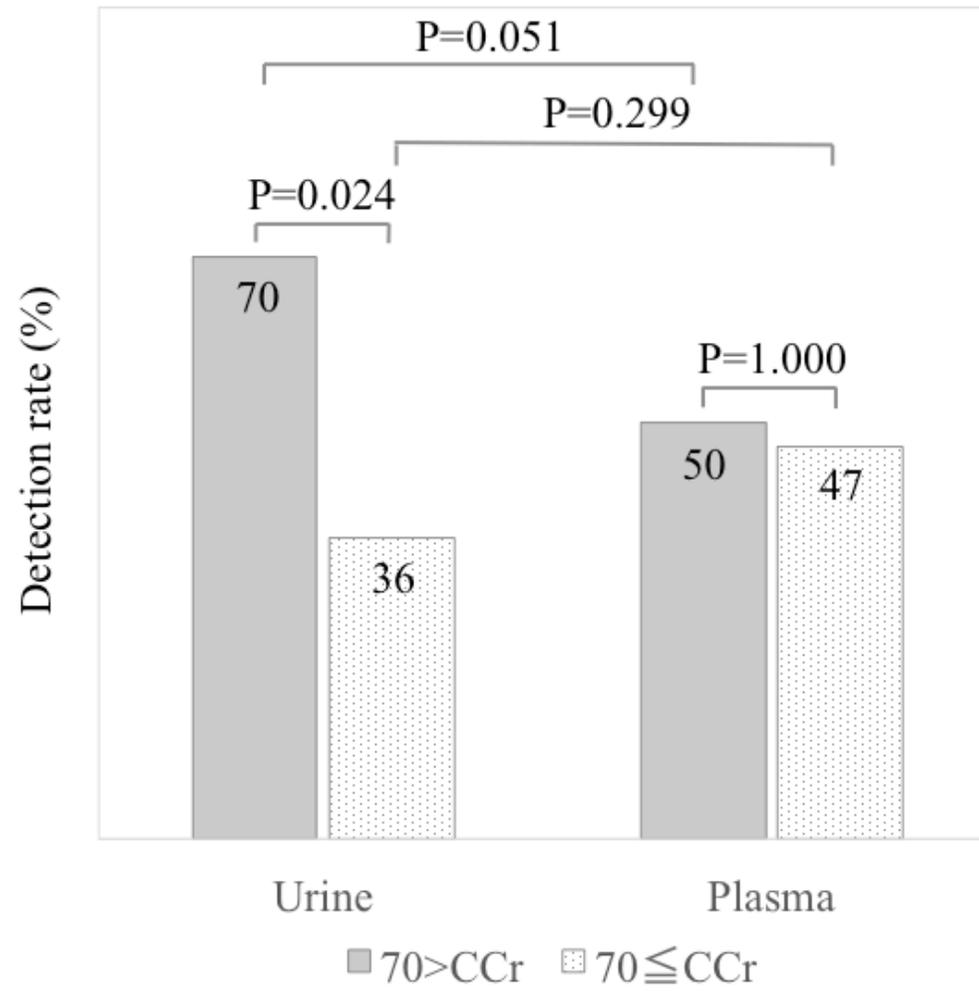


Figure 3

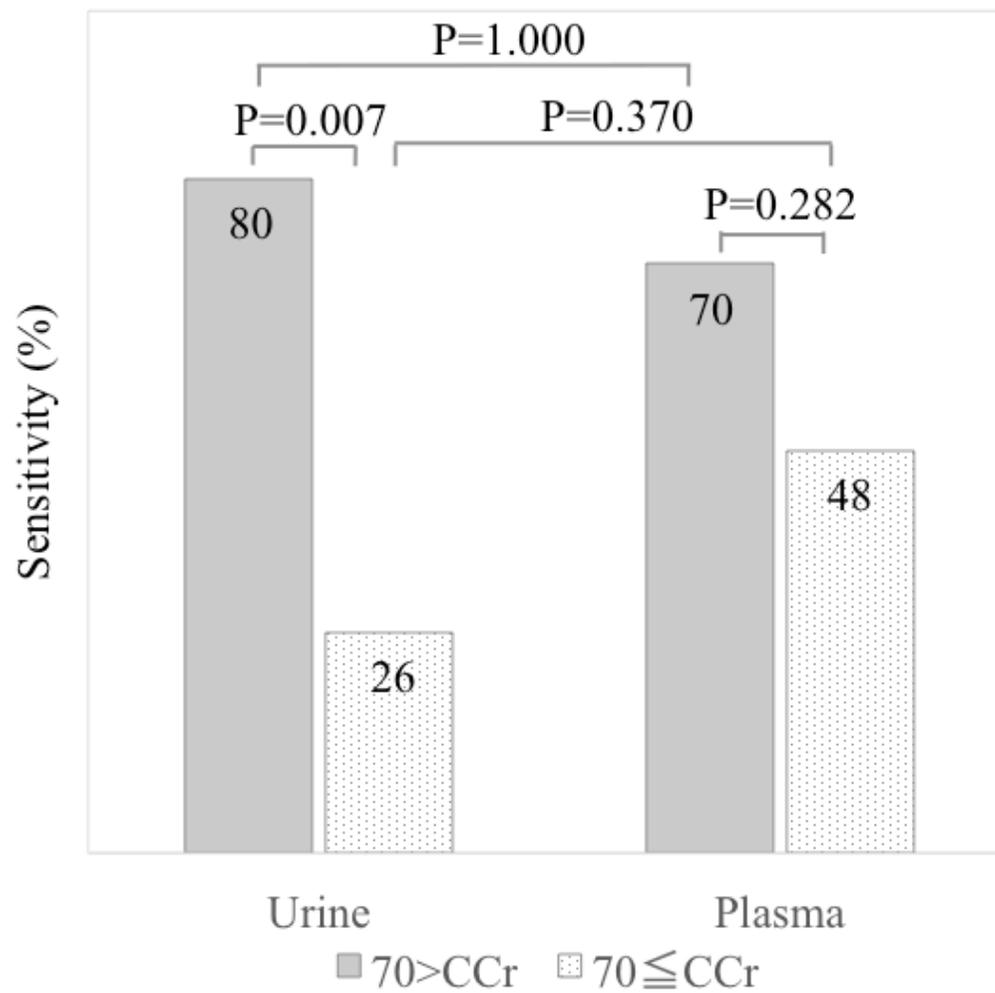


Figure 4

