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Expanding the Non-Invasive Diagnosis of Acute Rejection in Kidney Transplants Through Detection of Donor-Derived DNA in Urine: Proof-of-Concept Study

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Background: Approximately 10%–20% of kidney transplant (KT) recipients suffer from acute rejection (AR); thus, sensitive and accurate monitoring of allograft status is recommended. We evaluated the clinical utility of donor-derived DNA (dd-DNA) detection in the urine of KT recipients as a non-invasive means for diagnosing AR.

Methods: Urine samples serially collected from 39 KT recipients were tested for 39 single-nucleotide variant loci selected according to technical criteria (i.e., high minor allele frequency and low analytical error) using next-generation sequencing. The fraction of dd-DNA was calculated and normalized by the urine creatinine (UCr) level (%dd-DNA/UCr). The diagnostic performance of %dd-DNA/UCr for AR was assessed by ROC curve analysis.

Results: There was an increasing trend of %dd-DNA/UCr in the AR group before subsequent graft injury, which occurred before (median of 52 days) histological rejection. The serum creatinine (SCr) level differed significantly between the AR and non-AR groups at two and four months of follow-up, whereas %dd-DNA/UCr differed between the groups at six months of follow-up. The combination of %dd-DNA/UCr, SCr, and spot urine protein (UPtn)/UCr showed high discriminating power, with an area under the ROC curve of 0.93 (95% confidence interval: 0.81–1.00) and a high negative predictive value of 100.0%.

Conclusions: Although the dd-DNA–based test cannot eliminate the need for biopsy, the high negative predictive value of this marker could increase the prebiopsy probability of detecting treatable injury to make biopsy an even more effective diagnostic tool.

Key Words: Kidney transplantation, Acute rejection, Donor-derived DNA, Single-nucleotide variants, Next-generation sequencing, Urine

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INTRODUCTION

Kidney transplantation (KT) is the preferred treatment for patients with end-stage renal disease (ESRD). Although KT is a life-saving treatment, transplant recipients require lifelong follow-up, with intensive surveillance of allograft function. Approximately 10% and 20% of KT recipients suffer from acute rejection (AR), which is a major risk factor of graft failure [1].

Diagnostic biopsies are performed in cases with a strong clinical suspicion of AR, which mainly depends on the deterioration

of graft function determined as the estimated glomerular filtration rate (eGFR) measured in terms of serum creatinine (SCr) levels [2]. However, the level and rate of SCr change poorly predict graft failure, since the deterioration of kidney function follows graft injury [3]. Moreover, alterations in SCr levels are not specific, as they may also indicate an intrinsic process such as renal artery stenosis, recurrence of original disease, a transient process, or AR [4]. However, the current strategies for monitoring graft dysfunction are not sufficient for indicating the need for biopsy since up to 10.8% of grafts have normal histological results [5]. Therefore, a novel strategy is needed to decide whether to perform diagnostic biopsy in a timely manner.

Surrogate markers such as transcriptomic molecular profiles related to graft injury have been evaluated for the diagnosis of AR [6–9]. Although these markers can provide rich biological information, the degradative nature of RNA is a major barrier to their widespread adoption for clinical diagnosis [10]. In addition, these markers cannot accurately discriminate between various origins of damage, since they can be released from a remnant kidney or can be due to kidney-intrinsic etiologies [11].

Donor-derived DNAs (dd-DNAs) exist as extracellular cell-free DNA (dd-cfDNA) in the recipient or as an intracellular component of a donor cell (cellular dd-DNA), and both forms are likely to be released from necrotic or apoptotic cells in a transplanted organ [12, 13]. As the levels of dd-DNAs increase when an allograft is damaged by rejection or viral infection, they can be used as markers for graft injury [14, 15]. To distinguish dd-DNA from recipient DNA, detection of autosomal single-nucleotide variants (SNVs), given their wide range of uses, increases the discriminating power of dd-DNAs [13, 16].

Since graft cells or DNA can gain access to the urinary space, urine represents an appropriate sample type to investigate intragraft events [17]. Therefore, the urine of KT recipients may serve as a form of liquid biopsy, offering a truly non-invasive diagnostic method. Along with urinary tubule protein marker levels, the urinary dd-DNA level may increase after graft injury [18].

In this study, we evaluated the utility of urinary dd-DNA combined with other laboratory parameters to guide the timeliness of diagnostic biopsy. We evaluated clinical characteristics associated with outcomes and serially increasing levels of urinary dd-DNAs by multiplexing 39 autosomal informative SNVs identified through next-generation sequencing (NGS), and compared the time of urinary dd-DNA to increase with the time point of histological AR.

MATERIALS AND METHODS

Study population and samples

Forty-three patients with ESRD who had undergone scheduled KT from related or unrelated living donors from December 2014 to June 2015 at Severance Hospital, Seoul, Korea, were included in this observational prospective (sample collection and tests were performed the day before KT) and retrospective (clinical data were collected from medical records) study. Sample size was calculated based on the formula described by Buderer [19], with a maximum clinically acceptable two-tailed 95% confidence interval (CI) width of 0.1, estimated disease prevalence of 0.2, expected sensitivity of 0.9, and expected specificity of 0.9. Blood samples (>3 mL) were collected before KT to assess informative SNVs, whereas post-KT urine (>10 mL) and blood samples (>3 mL) were prospectively collected at the time of serial follow-up visits at 1 week; 2 weeks; and 1, 2, 4, and 6 months, as regular intervals; and at the time of biopsy. However, collection was discontinued at the time of AR detection (Fig. 1).

Patients' demographic and clinical data were extracted by retrospectively reviewing electronic medical records. AR was diagnosed by graft biopsy, which was performed for patients with deteriorating graft function. Histological diagnosis of AR was made by a single pathologist according to the Banff 2007 criteria [20]. The Institutional Review Board (IRB) of Severance Hospital approved this study (IRB 2015-1707-001). Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Sample collection and processing

First morning concentrated midstream urine samples (10–15 mL) were collected in sterile containers. Within 2 hours of collection, the samples were centrifuged at 2,000×*g* for 20 minutes at room temperature (20–25°C). To acquire urinary cellular dd-DNA and to avoid its degradation, the supernatant was separated from the urine pellet containing cells and cell debris. The cell pellet was transferred to a 1.5-mL microcentrifuge tube containing 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) and stored at –80°C for further analysis. SCr, spot urine protein (UPtn), and urine creatinine (UCr) levels were measured using a Beckman Coulter AU680 analyzer (Beckman Coulter, Fullerton, CA, USA).

DNA extraction and multiplex PCR targeted amplicon sequencing

Genomic DNA was extracted from urinary cell pellets using QIA-

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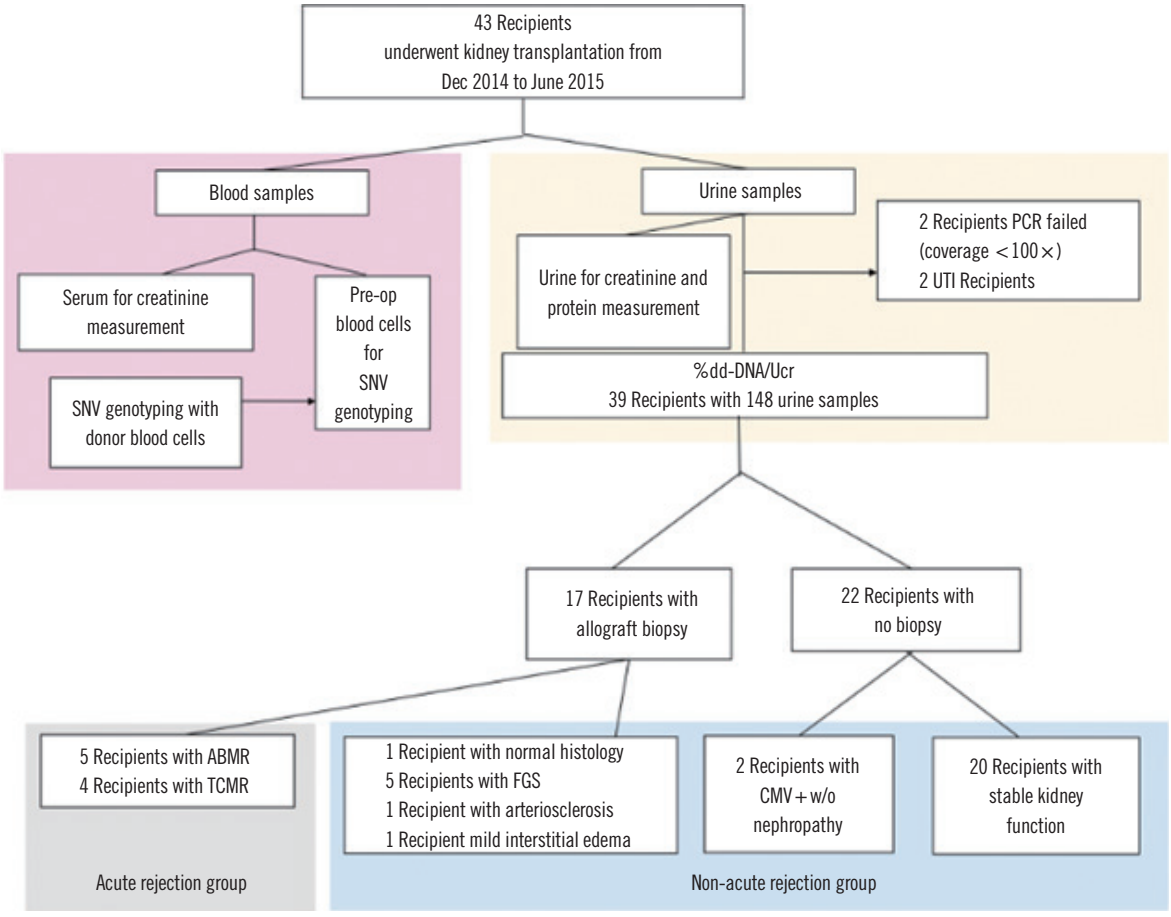

Fig. 1. Flow diagram of this study. A total of 39 recipients were evaluated for %dd-DNA/UCr. Two of four recipients with inadequate PCR results and two other recipients with bacterial UTIs were excluded.
Abbreviations: dd-DNA, donor-derived DNA; UCr, urine creatinine; UTIs, urinary tract infections; SNV, single-nucleotide polymorphism; ABMR, antibody-mediated rejection; TCMR, T cell-mediated rejection; FGS, focal glomerulosclerosis; CMV, cytomegalovirus.

amp MinElute Column kit (Qiagen, Hilden, Germany) according to the standard procedure. For library construction, the optimal input DNA was 20 ng according to the manufacturer's protocol; samples of two recipients with a urinary DNA level <10 ng/μL or failed amplification (PCR-failed samples), and samples of two recipients with urinary tract infections (UTIs) were excluded.

Thirty-nine SNVs were selected according to the following criteria: minor allele frequency (MAF) >0.4, known low polymerase error, high coverage (>1,000 counts) in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), low linkage (>500-kb apart), no more than one additional SNV with MAF >0.1 in the amplicon, and no known association with disease. In addition, targets of interest were selected if the adjacent allele was less than 5-bp away with a MAF of >0.5 to visually detect the sequencing bias.

Amplicons were indexed with dual-matched adapters (i5 and i7) with Unique Molecular Indices (UMI) designed to significantly reduce index misassignment. All 39 libraries were sequenced on a NextSeq550 flowcell (Illumina, San Diego, CA, USA) using a V3 NextSeq550 sequencing kit (Illumina). Further data analysis, including quality check, sequence alignment, and variant calling, were performed with a customized bioinformatics pipeline.

Analytical performance of SNV markers

The limit of detection (LoD) for dd-DNA measurement was estimated by serially diluting equimolar amounts of DNA extracted from the whole blood of two unrelated individuals (normal controls) using dilution factors of 50%, 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001%. The dilution factors were transformed to log base 10 to warrant low-level values. The estimation was performed using fragmented DNA at a total input mass of 20 ng. We used the linear regression model to fit the data to the regression line and G-test to determine the appropriateness of the model [21].

Table 1. Informative SNVs and variant allele frequency distributions of 39 recipients according to days post-transplantation

Recipient number	N informative SNVs*	Outcome	Days after transplantation	% VAF of informative SNVs	Mean depth
KT01	11	Non-AR	28	0.315	8,156
			105	0.262	13,898
			168	1.476	10,231
KT02	7	Non-AR	168	0.885	11,317
			196	6.545	9,866
KT03	11	AR	14	2.008	7,338
			21	15.189	234
			35	1.798	12,158
KT04	10	Non-AR	28	7.863	3,872
			56	14.164	1,481
			112	19.335	290
			168	28.726	953
			196	60.878	798
KT05	10	AR	14	18.449	687
			28	60.199	859
			98	41.693	1,059
KT06	6	Non-AR	21	0.15	1,170
			84	0.22	1,181
			224	27.358	379
KT07	16	AR	7	10.279	6,257
			28	14.012	3,574
			56	16.531	2,002
			84	6.016	10,501
KT08	13	AR	14	71.87	215
			49	74.835	7,369
			140	74.182	6,140
			168	87.5	667
KT09	12	Non-AR	7	22.247	200
			14	3.045	9,065
KT10	6	Non-AR	14	12.29	253
			28	3.69	5,120
			168	60.259	953
			196	50.038	2,836
KT11	7	Non-AR	56	15.27	198
			168	53.733	197
			196	66.709	570
KT12	13	Non-AR	21	0.747	16,881
			63	1.112	21,639
			112	0.541	17,665
			196	1.772	7,821
KT13	3	Non-AR	21	7.485	2,917
			98	1.096	12,332
			140	6.866	6,218
KT14	15	Non-AR	7	6.55	7,994
			35	60.226	184
			84	18.166	1,129
			196	71.989	299
KT15	4	Non-AR	7	9.766	1,083
			14	1.482	3,149
			21	1.729	9,980
			28	1.589	16,213
			84	1.274	10,194
			112	4.617	5,072
			168	3.48	10,687
			196	3.642	9,194
KT16	5	AR	7	47.212	4,863
			14	30.175	11,197
			28	38.849	326
KT17	6	Non-AR	7	14.444	1,131
			14	0.877	10,340
			21	1.427	8,684
			28	0.545	9,756
			56	0.279	8,548
KT18	14	Non-AR	14	14.217	1,374
			21	7.008	2,124
			56	6.775	986
			168	10.675	544
KT19	10	Non-AR	21	7.199	6,390
			28	15.113	2,201
			56	21.449	9,504
			140	30.468	1,179
			168	24.242	690
KT20	6	Non-AR	14	5.373	7,910
			28	2.724	6,112
			56	10.671	4,680
			84	7.544	1,307
KT21	11	Non-AR	14	1.104	6,741
			21	0.773	6,372
			28	1.302	6,693
			56	2.892	3,462
			112	1.416	1,820
			140	2.39	1,972
			168	2.231	1,138

(*Continued to the next*)

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Table 1. Continued

Recipient number	N informative SNVs*	Outcome	Days after transplantation	% VAF of informative SNVs	Mean depth
KT22	10	AR	7	37.995	595
			28	2.386	7,236
			35	5.667	1,772
KT23	7	Non-AR	21	57.325	443
			28	9.601	694
			56	44.057	3,159
			140	12.425	1,103
			168	26.747	164
KT24	12	Non-AR	7	0.779	7,900
			14	20.215	212
KT25	6	Non-AR	21	31.853	1,733
			28	76.37	1,202
			56	39.785	529
			98	11.965	1,084
			140	34.996	155
			168	67.116	358
KT26	14	Non-AR	7	4.451	2,753
			21	3.166	6,206
			28	1.564	577
			56	7.804	5,964
			168	6.526	3,915
			196	27.5	117
KT27	13	Non-AR	21	75.125	4,275
			28	70.905	861
			56	47.568	179
			84	69.772	575
			112	62.085	319
			140	55.039	1,018
KT28	18	Non-AR	7	73.252	4,460
			21	51.656	298
			28	71.759	2,175
			49	68.115	2,853
			84	69.13	2,829
			112	70.321	1,243
			140	64.442	6,659
KT29	16	Non-AR	7	81.141	1,448
			14	87.269	156
KT30	19	Non-AR	7	59.978	215
			21	83.587	607
KT31	10	Non-AR	7	3.449	15,343
			14	3.683	2,645
KT32	12	Non-AR	7	1.42	4,315
			14	9.069	314
KT33	11	AR	7	22.03	7,999
			21	5.062	19,788
			56	4.564	20,496
KT34	7	Non-AR	14	9.213	293
			21	6.532	13,266
			28	9.941	2,271
			56	10.483	5,422
KT35	9	Non-AR	14	10.816	517
			28	11.187	2,630
			56	23.901	177
			84	17.485	271
KT36	14	Non-AR	14	27.191	814
			28	14.659	1,620
			56	18.461	201
KT37	13	AR	14	14.544	2,919
			21	14.918	3,103
			56	28.527	1,219
			63	22.442	2,787
KT38	10	Non-AR	7	45.796	156
			28	14.405	159
KT39	10	AR	14	2.542	7,328
			28	49.321	402
			35	4.727	499

(*Continued to the next*)

*The following 39 SNV markers were used for the chimerism calculation: rs3738561, rs6480497, rs4757113, rs7983800, rs3745331, rs10426644, rs2540307, rs1358833, rs62270249, rs1436501, rs9386037, rs2159478, rs11023112, rs6589967, rs8022985, rs1202017, rs645107, rs6921313, rs4072990, rs6676162, rs72735619, rs11187560, rs10832201, rs7950719, rs6590643, rs1731550, rs10777988, rs4496026, rs12327492, rs1348784, rs281544, rs6445350, rs3819864, rs6863833, rs1423013, rs1561681, rs73230060, rs6995506, and rs16904057.
Abbreviations: SNV, single-nucleotide variant; VAF, variant allele frequency; AR, acute rejection, including acute antibody-mediated rejection and T cell-mediated rejection.

Linear regression analysis indicated a good linear correlation (R^2=0.89, P=0.001), and the LoD was validated from 0.01% of the NGS results (% NGS=1.38×% theoretical dilution–0.33). The mean number of informative SNVs per patient was 10.4, with actual numbers ranging from 3 to 19. The average sequencing depth per sample was 4,199.5±4,749.8 reads. The informative markers were distributed across 18 chromosomes, with a mean product size of 83.6±6.2 bp and a mean distance be-

tween markers on the same chromosome of 99.8±67.9 Mb (Table 1).

Measurement of dd-DNA

The fraction of dd-DNA (%dd-DNA) was calculated by dividing the read numbers of variant sequences corresponding to a donor genotype by the total coverage numbers of target sequences in each informative SNV. Averaging percentages were calculated for all informative SNVs. If a donor-specific genotype was heterozygous, recipient-specific variant read numbers were multiplied by two based on the method described in our previous study [22]. Background levels of an alternate allele resulting from an amplification or sequencing error were subtracted from the alternate allele frequency for each SNV site. The calculated %dd-DNA was normalized against the UCr level of a sample. The maximal %dd-DNA/UCr was defined as the highest %dd-DNA/UCr level among serial %dd-DNA/UCr values measured for each recipient (in both the AR and non-AR groups) at a certain time point and as indicative of the occurrence of severe molecular injury.

Statistical analysis

Continuous variables with non-normal distribution, including the age and body mass index (BMI) of the recipient and donor, allograft length, number of mismatched HLA types, and average percentage of screened panel-reactive antibody (PRA), are presented as median (interquartile range [IQR]). Continuous values such as %dd-DNA/UCr and SCr levels between the two groups (AR and non-AR) were compared based on the Mann-Whitney rank-sum test and are presented as median (range). Categorical variables, including the sex of recipient and donor, relation between the recipient and donor, and ABO compatibility, are presented as numbers and percentages. These variables were compared using either the chi-square test or Fisher's exact test, as appropriate. The diagnostic performance of %dd-DNA/UCr (maximal %dd-DNA/UCr) was evaluated by receiver operating characteristic (ROC) curve analysis, and the glm function for modeling and visualization of plots was used in the R software, version 3.5.2, 64-bit (R Foundation for Statistical Computing, Vienna, Austria). The sensitivity and specificity in the ROC curve analysis were estimated using the Youden index. $P<0.05$ was considered statistically significant.

Table 2. Demographic data of the 39 donors and recipients

Characteristics*	AR group (N=9)	Non-AR group (N=30)	$P^{\dagger}$
Age at transplantation (yr)			
Recipient	49.0 (44.0–53.0)	44.0 (36.0–54.0)	0.385
Donor	47.0 (45.0–53.0)	40.0 (33.0–49.0)	0.054
Sex			
Female/Male	2/7 (28.6%)	9/21 (42.9%)	0.595
Body mass index (kg/m^2)			
Recipient	22.4 (20.6–24.2)	22.5 (18.7–25.9)	0.958
Donor	22.3 (21.0–23.5)	22.5 (20.4–24.7)	0.741
Allograft length (cm)	10.5 (10.3–11.4)	11.0 (10.3–11.3)	0.446
Donor type (living)			
Genetically related	2/9 (22.2%)	22/30 (73.3%)	**0.001**
Parent	0	12	
Sibling	2	10	
Genetically unrelated (Spouse)	7/9 (77.8%)	8/30 (26.7%)	
Tacrolimus trough level (ng/mL)	3.60 (3.10–4.85)	4.73 (3.8–5.82)	0.110
ABO incompatibilities, N (%) (donor→recipient)	1 (11.1%)	10 (33.3%)	0.421
HLA mismatch (HLA-A, B, DR)	5 (3–6)	3 (2–4)	**0.044**
PRA Screening (%) (average)	0.0 (0.0–0.0)	0.0 (0.0–0.75)	0.208

*All data are shown as median (IQR) unless otherwise indicated (i.e., N, %); †Significant *P* values are in bold.
Abbreviations: AR, acute rejection, including acute antibody-mediated rejection and T cell- mediated rejection; PRA, panel-reactive antibody; SD, standard deviation.

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RESULTS

Patient characteristics and %dd-DNA/UCr

AR was more likely to occur in recipients who had received a transplant from an unrelated donor (P=0.001) and had a greater number of mismatched HLA types (P=0.044). There were no significant differences between the AR and non-AR groups in the age at KT (recipient and donor), sex of the recipient, BMI (recipient and donor), allograft length, tacrolimus trough level, ABO incompatibility, and average % PRA (Table 2).

AR diagnosis and %dd-DNA/UCr

AR was diagnosed in nine recipients. Five recipients had acute antibody-mediated rejection and four had acute T-cell mediated rejection (Table 3). AR occurred at a median of 63 (47.5–111.5) days after the KT. Eight of the nine recipients developed rejection in the first four months after KT.

There was wide intra-recipient variation of %dd-DNA/UCr in the urine, even when considering all 56 samples of the 20 recipients in the non-AR group whose allograft remained stable (dd-DNA/UCr range: 0.10%–48.92%), suggesting that there might be a response to other subclinical acute graft injuries. However, there was an increasing trend of %dd-DNA/UCr in the AR group before subsequent graft injury. The elevation of %dd-DNA/UCr occurred from 85 days to 12 days earlier (median of 52 days) than histological rejection.

The SCr differed significantly between the AR and non-AR groups at 2 and 4 months (P<0.05), whereas %dd-DNA/UCr differed significantly at 6 months of the follow-up period (Fig. 2)

Table 3. Histological results for patients with AR

Recipient number	Gender	Age (yr)	Relationship to donor	Histological diagnosis (Banff 2007)	Time to rejection (days)
KT03	F	28	Spouse	ABMR, grade II and TCMR, type IB (g0, t3, i3, v0, cg0, ct0, ci0, cv0, mm0, ah0, ptc2)	33
KT05	M	56	Spouse	Suspicious for TCMR (g0 t1 i1 v0 cg0 ct0 ci0 cv0 ah0 mm0 ptc0)	113
KT07	M	49	Spouse	TCMR, type IB (g0 t3 i3 v0 cg0 ct0 ci0 cv0 ah0 mm0 ptc2)	110
KT08	M	61	Spouse	Suggestive of ABMR (g0 t1 i1 v0 cg0 ct0 ci0 cv0 ah1 mm0 ptc0)	163
KT16	M	49	Sibling	ABMR (g3 t0 i3 v2 cg0 ct0 ci0 cv0 ah0 mm0 ptc2)	46
KT22	F	40	Sibling	ABMR, grade II (g3 t0 i0 v0 cg0 ct0 ci0 cv0 ah0 mm0 ptc1)	70
KT33	M	53	Spouse	TCMR, type IIA (g0 t2 i2 v1 cg0 ct0 ci0 cv0 mm0 ah0 ptc0)	63
KT37	M	46	Spouse	ABMR, type II (g0 t1 i2 v0 cg0 ct0 ci0 cv0 mm0 ah0 ptc3)	56
KT39	M	44	Spouse	TCMR, type IIA (g1 t3 i3 v2 cg0 ct0 ci0 cv0 mm0 ah0 ptc2)	49

Abbreviations: KT, kidney transplantation; AR, acute rejection; ABMR, antibody-mediated rejection; TCMR, T-cell mediated rejection.

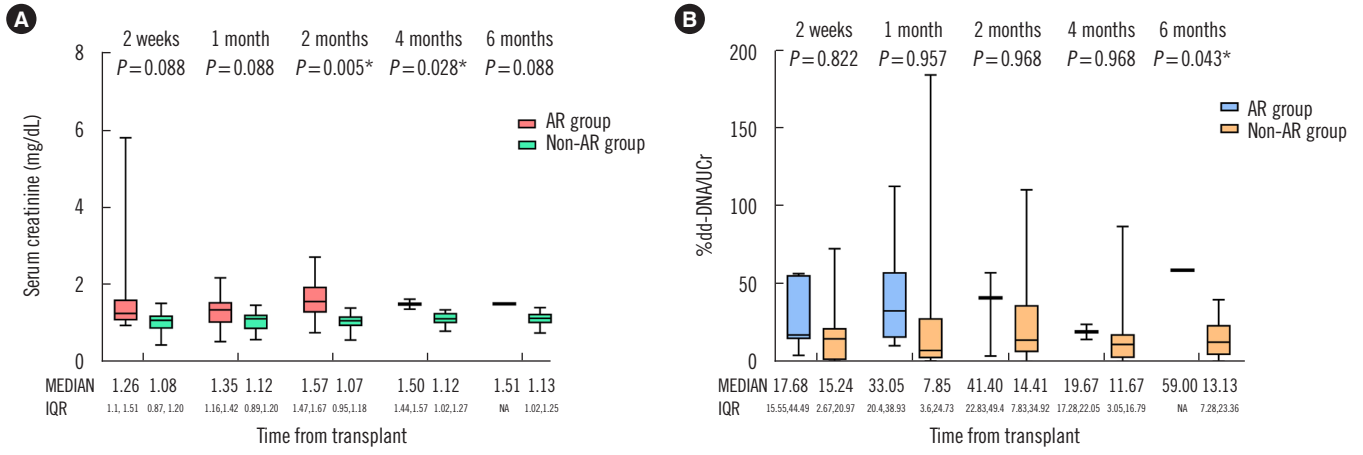

Fig. 2. Comparison of SCr and %dd-DNA)/UCr between groups (AR vs. Non-AR). (A) SCr and (B) %dd-DNA)/UCr between the AR and non-AR groups at different time points (at 2 weeks 2, months 1, 2, 4, and 6) after transplantation. *P* values are presented above the box plots. Median (interquartile range [IQR]) values are presented at the bottom of graphs, outside of the box plots.
Abbreviations: SCr, serum creatinine; dd-DNA, donor-derived DNA; UCr, urine creatinine; AR acute rejection; Non-AR, non-acute rejection.

Table 4. Comparison of AUC values of ROC curves for % maximal dd-DNA/UCr, SCr, UPtn/UCr, and their combinations

	AUC (95% CI)	Cut-off*	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
%dd-DNA/UCr†	0.58 (0.42–0.75)	47.50%	53.6	77.8	15.2	95.7
SCr (mg/dL)	0.79 (0.60–0.98)	1.3	78.3	83.3	0.58	96.6
UPtn/UCr	0.78 (0.57–0.98)	58.4	57.9	100.0	33.3	100.0
SCr and UPtn/UCr	0.91 (0.76–1.00)	0.2	86.7	100.0	60.0	97.7
%dd-DNA/UCr, SCr and UPtn/UCr	0.93 (0.81–1.00)	0.2	86.7	100.0	60.0	100.0

*The cut-off values for %dd-DNA/UCr, SCr, and UPtn/UCr were individually selected to yield the highest Youden index, whereas the cut-offs for their combinations were determined using a non-parametric general linear model. †The diagnostic performance of %dd-DNA/UCr, evaluated by ROC curve analysis, were values of maximal %dd-DNA/UCr, which is defined as the highest %dd-DNA/UCr level among serial %dd-DNA/UCr values measured for each recipient (in both the AR and non-AR groups) at a certain time point.
Abbreviations: AUC, area under the curve; ROC, receiver operating characteristic; CI, confidence interval; dd-DNA, donor-derived DNA; UCr, urine creatinine; SCr, serum creatinine; UPtn, spot urine protein.

Diagnostic performance of dd-DNA for AR

The area under the ROC curve (AUC) for discriminating AR from non-AR groups was poor for %dd-DNA/UCr alone, which cannot be used to establish the diagnosis of AR. The AUC of SCr alone and UPtn/UCr alone were higher than that of %dd-DNA/UCr alone. The discriminating power improved with the combination of %dd-DNA/UCr, SCr, and UPtn/UCr (0.93), which was similar to that observed with the combination of SCr and UPtn/UCr (0.91).

The positive predictive value (PPV) of %dd-DNA/UCr alone compared to SCr alone showed improved performance (15.2% vs. 0.58%) when screening AR; however, the performance was still inferior to that of UPtn/UCr (33.3%). The PPV of %dd-DNA/UCr alone improved with the combination of SCr and UPtn/UCr to 60.0%. However, a high negative predictive value (NPV) was found for %dd-DNA/UCr, SCr, and Uptn/UCr, both individually and in combination (Table 4).

DISCUSSION

In this study, the clinical performance of %dd-DNA/UCr for AR diagnosis was assessed and compared with the performance of standard diagnostic tools such as allograft biopsy and traditional analyses of laboratory parameters, including SCr and UPtn. We applied 39 highly discriminative autosomal SNVs with analytic accuracy. We suggest autosomal SNVs as the most appropriate markers of allograft rejection in clinical practice because analysis of the Y chromosome is only suitable for female KT recipients from male donors, and more than half of KT in Korea are from genetically related donors [23, 24].

Several studies have employed methods that quantify dd-DNA, including quantitative PCR, digital-droplet PCR (dPCR), and targeted NGS [16]. dPCR is a sensitive and cost-effective method to quantify circulating nucleic acids; however, depending on the instrument, it is susceptible to poor test design, leading to cross-reactivity and false positives [25]. NGS also has the potential to introduce biases such as pre-amplification of dd-DNA [26]. Thus, we selected targets of interest with a GC content <61% and adjacent alleles within a 5-bp region having an MAF of >0.5 to visually detect any sequencing bias. Since NGS-based multiplex platforms are feasible for hundreds of primer pairs and their cost is continuously reducing, their widespread utility is expected, especially for the monitoring of multiple organ transplantations from different donors.

Numerous types of nucleic acids can be measured in the urine, including cfDNA, cellular DNA, and RNAs such as microRNAs, long non-coding RNAs, and mRNAs. The fraction of cellular DNA in the urine is far greater than that of cfDNA, which occurs in donor-derived vascular/tubular cells and lymphocytes in the urine of KT recipients [18, 27]. Many clinical studies have evaluated the diagnostic value of dd-DNA, especially in the form of plasma and urinary cell-free dd-DNA (dd-cfDNA), for the prediction of AR [14, 15, 28–30]. The levels of dd-cfDNA were shown to be sensitive to graft injury, with unstable kinetics in the early post-transplantation phase [31]. This means that their fluctuations need to be interpreted in conjunction with other clinical and laboratory parameters [16]. Moreover, the low level of cfDNA is problematic, as many molecular techniques require higher DNA amounts, and contamination by cellular DNA or PCR inhibitors affects NGS performance [32]. Therefore, we concluded that cellular dd-DNA is more suitable for multiplex PCR enrichment for urine samples of KT recipients, and the abundant cellular dd-DNA is more adequate to conduct monitoring.

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We excluded patients with UTI to minimize confounding factors. High intra-recipient variation, with dd-DNA/UCr ranging from 0.10% to 48.92%, was observed in non-AR recipients. This result was not surprising because cellular dd-DNA reflects tissue breakdown due to injury in a donor organ, and regeneration of a transplanted kidney is a normal physiological process after transplantation [33, 34]. However, the observed %dd-DNA/UCr fluctuation could not be histologically explained, since a protocol biopsy was not obtained at each time point.

Increased %dd-DNA/UCr before AR was observed and was significantly discriminable from that in the non-AR group at 6-month follow-up, whereas a difference in SCr levels was observed between the AR and non-AR groups at the 2-month and 4-month follow-ups. The inclusion of %dd-DNA/UCr with SCr and UPtn/UCr did not affect the diagnostic performance , which may be due to relatively scant number of urine samples available for %dd-DNA/UCr measurements owing to the unpredictable timing of AR and biological variation among urine samples. However, molecular injury, represented as the maximal %dd-DNA/UCr, occurred earlier than clinical or histological AR, with a median of 52 days, which implies that %dd-DNA/UCr is a sensitive marker for AR.

This study has several limitations. First, we did not perform a protocol biopsy for surveillance and the total number of biopsy-confirmed AR allografts was small. Therefore, we could not estimate the baseline %dd-DNA/UCr for all biopsy-confirmed stable allografts. Second, only living-donor KT recipients were included in the study since part of the samples and consent had to be obtained before KT. Since the majority of transplantations use organs derived from deceased donors, the translation of our results to the deceased donor pool remains to be confirmed.

To the best of our knowledge, this is the first study to examine cellular dd-DNA from the urine samples of KT recipients using an SNV-based NGS approach and to evaluate the diagnostic performance of this approach with adjunctive biomarkers. Our results might help patients identify a possibility of transplant rejection before deciding on proceeding with a kidney biopsy. Informed biopsy decisions are needed to reduce morbidity and increase the cost-effectiveness of transplant recipient surveillance. Our strategy would be especially useful for patients who are on anticoagulation therapy or have other reasons to avoid biopsy. Based on our research, additional studies regarding analytical standardization and validation of urinary dd-DNA are needed for its clinical application.

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AUTHOR CONTRIBUTIONS

Kim J participated in research design and in the writing of the paper. Kim DM participated in the performance of the research. Park YJ participated in the acquisition of data. Lee ST contributed provided technical and analytical support. Kim HS participated in advising research methodology. Kim MS participated in research design and collection of samples. Kim BS participated in critically revising the study. Choi JR supervised the full study and acquired financial support.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Harada KM, Mandia-Sampaio EL, de Sandes-Freitas TV, Felipe CR, Park SI, Pinheiro-Machado PG, et al. Risk factors associated with graft loss and patient survival after kidney transplantation. Transplant Proc 2009;41:3667-70.
2. Thomas ME, Blaine C, Dawnay A, Devonald MA, Ftouh S, Laing C, et al. The definition of acute kidney injury and its use in practice. Kidney

Int 2015;87:62-73.
3. Lachenbruch PA, Rosenberg AS, Bonvini E, Cavaillé-Coll MW, Colvin RB. Biomarkers and surrogate endpoints in renal transplantation: present status and considerations for clinical trial design. Am J Transplant 2004;4:451-7.
4. Josephson MA. Monitoring and managing graft health in the kidney transplant recipient. Clin J Am Soc Nephrol 2011;6:1774-80.
5. Devadass CW, Vanikar AV, Nigam LK, Kanodia KV, Patel RD, Vinay KS, et al. Evaluation of renal allograft biopsies for graft dysfunction and relevance of C4d staining in antibody mediated rejection. J Clin Diagn Res 2016;10:EC11-5.
6. Held PJ, McCormick F, Ojo A, Roberts JP. A cost-benefit analysis of government compensation of kidney donors. Am J Transplant 2016;16:877-5.
7. Shahbaz SK, Pourrezagholi F, Barabadi M, Foroughi F, Hosseinzadeh M, Ahmadpoor P, et al. High expression of TIM-3 and KIM-1 in blood and urine of renal allograft rejection patients. Transpl Immunol 2017;43-44:11-20.
8. Lorenzen JM, Volkmann I, Fiedler J, Schmidt M, Scheffner I, Haller H, et al. Urinary miR-210 as a mediator of acute T-cell mediated rejection in renal allograft recipients. Am J Transplant 2011;11:2221-7.
9. Lorenzen JM, Schauerte C, Kölling M, Hübner A, Knapp M, Haller H, et al. Long noncoding RNAs in urine are detectable and may enable early detection of acute T cell-mediated rejection of renal allografts. Clin Chem 2015;61:1505-14.
10. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002;161:1961-71.
11. Vaidya VS, Ferguson MA, Bonventre JV. Biomarkers of acute kidney injury. Annu Rev Pharmacol Toxicol 2008;48:463-93.
12. Lo YM, Tein MS, Pang CC, Yeung CK, Tong KL, Hjelm NM. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. Lancet 1998;351:1329-30.
13. Beck J, Bierau S, Balzer S, Andag R, Kanzow P, Schmitz J, et al. Digital droplet PCR for rapid quantification of donor DNA in the circulation of transplant recipients as a potential universal biomarker of graft injury. Clin Chem 2013;59:1732-41.
14. Whitlam JB, Ling L, Skene A, Kanellis J, Ierino FL, Slater HR, et al. Diagnostic application of kidney allograft-derived absolute cell-free DNA levels during transplant dysfunction. Am J Transplant 2019;19:1037-49.
15. Bloom RD, Bromberg JS, Poggio ED, Bunnapradist S, Langone AJ, Sood P, et al. Cell-free DNA and active rejection in kidney allografts. J Am Soc Nephrol 2017;28:2221-32.
16. Knight SR, Thorne A, Lo Faro ML. Donor-specific cell-free DNA as a biomarker in solid organ transplantation. A systematic review. Transplantation 2019;103:273-83.
17. Nissaisorakarn V, Lee JR, Lubetzky M, Suthanthiran M. Urine biomarkers informative of human kidney allograft rejection and tolerance. Hum Immunol 2018;79:343-55.
18. Zhang Z, Ohkohchi N, Sakurada M, Mizuno Y, Miyagi S, Satomi S, et al. Analysis of urinary donor-derived DNA in renal transplant recipients with acute rejection. Clin Transpl 2002;16(S8):45-50.
19. Buderer NMF. Statistical methodology: I. Incorporating the prevalence of disease into the sample size calculation for sensitivity and specificity. Acad Emerg Med 1996;3:895-900.
20. Solez K, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, et al. Banff 07 classification of renal allograft pathology: updates and future directions. Am J Transplant 2008;8:753-60.
21. Jhang JS, Chang CC, Fink DJ, Kroll MH. Evaluation of linearity in the clinical laboratory. Arch Pathol Lab Med 2004;128:44-8.
22. Kim J, Hwang IS, Shin S, Choi JR, Lee ST. SNP-based next-generation sequencing reveals low-level mixed chimerism after allogeneic hematopoietic stem cell transplantation. Ann Hematol 2018;97:1731-4.
23. Sigdel TK, Vitalone MJ, Tran TQ, Dai H, Hsieh SC, Salvatierra O, et al. A rapid noninvasive assay for the detection of renal transplant injury. Transplantation 2013;96:97-101.
24. Chung BH, Jung MH, Bae SH, Kang SH, Hwang HS, Hyoung BJ, et al. Changing donor source pattern for kidney transplantation over 40 years: a single-center experience. Korean J Intern Med 2010;25:288-93.
25. Huggett JF, Cowen S, Foy CA. Considerations for digital PCR as an accurate molecular diagnostic tool. Clin Chem 2015;61:79-88.
26. Aird D, Ross MG, Chen WS, Danielsson M, Fennell T, Russ C, et al. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. Genome Biol 2011;12:R18.
27. Augustus E, Van Casteren K, Sorber L, van Dam P, Roeyen G, Peeters M, et al. The art of obtaining a high yield of cell-free DNA from urine. PloS One 2020;15:e0231058.
28. Grskovic M, Hiller DJ, Eubank LA, Sninsky JJ, Christopherson C, Collins JP, et al. Validation of a clinical-grade assay to measure donor-derived cell-free DNA in solid organ transplant recipients. J Mol Diagn 2016;18:890-902.
29. Jordan SC, Bunnapradist S, Bromberg JS, Langone AJ, Hiller D, Yee JP, et al. Donor-derived cell-free DNA identifies antibody-mediated rejection in donor specific antibody positive kidney transplant recipients. Transplant Direct 2018;4:e379.
30. Lee H, Park YM, We YM, Han DJ, Seo JW, Moon H, et al. Evaluation of digital PCR as a technique for monitoring acute rejection in kidney transplantation. Genomics Inform 2017;15:2-10.
31. Gielis EM, Beirnaert C, Dendooven A, Meysman P, Laukens K, De Schrijver J, et al. Plasma donor-derived cell-free DNA kinetics after kidney transplantation using a single tube multiplex PCR assay. PloS One 2018;13:e0208207.
32. Johansson G, Andersson D, Filges S, Li J, Muth A, Godfrey TE, et al. Considerations and quality controls when analyzing cell-free tumor DNA. Biomol Detect Quantif 2019;17:100078.
33. Hung ECW, Shing TKF, Chim SSC, Yeung PC, Chan RW, Chik KW, et al. Presence of donor-derived DNA and cells in the urine of sex-mismatched hematopoietic stem cell transplant recipients: implication for the transrenal hypothesis. Clin Chem 2009;55:715-22.
34. Franquesa M, Flaquer M, Cruzado JM, Grinyó JM. Kidney regeneration and repair after transplantation. Curr Opin Organ Transplant 2013;18:191-6.

https://doi.org/10.3343/alm.2021.41.5.469