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

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

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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), 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-
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].

**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.
| 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                    | 893        |
|                  |                   |         | 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                         | 22.479                    | 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      |

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

Table 1. Continued

| Recipient number | N informative SNVs* | Outcome | Days after transplantation | % VAF of informative SNVs | Mean depth |
|------------------|-------------------|---------|---------------------------|---------------------------|------------|
| 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                        | 18.165                    | 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      |
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      |

*The following 39 SNV markers were used for the chimerism calculation: rs3738561, rs6480497, rs4757113, rs7983800, rs3745331, rs10426644, rs2540307, rs1358833, rs62270249, rs1456501, rs9386037, rs2154798, rs11023112, rs6589967, rs8022985, rs1202017, rs6921313, rs4072990, rs6651612, rs72735619, rs11187560, rs10832201, rs7950719, rs6590643, rs1713550, rs10777988, rs4496026, rs12327492, rs1348784, rs281544, rs6445350, rs319864, rs6863833, rs1423013, rs1561681, rs73230060, rs6995506, and rs1690457.

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-

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between 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.

Table 2. Demographic data of the 39 donors and recipients

| Characteristics* | AR group (N = 9) | Non-AR group (N = 30) | P† |
|------------------|-----------------|----------------------|----|
| 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/m²) |   |                      |    |
| 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.

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.
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).

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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.

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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, 1 month, 2 months, 4 months, and 6 months) 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.
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 a 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.
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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