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Donor-derived Cell-free DNA Identifies Antibody-mediated Rejection in Donor Specific Antibody Positive Kidney Transplant Recipients

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Background. Elevated levels of donor-derived cell-free DNA (dd-cfDNA) in the plasma of renal allograft recipients indicates organ injury and an increased probability of active rejection. Donor-specific antibodies (DSA) to HLA antigens are associated with risk of antibody-mediated rejection (ABMR). This study assessed the combined use of dd-cfDNA and DSA testing to diagnose active ABMR. **Methods.** Donor-derived cell-free DNA was assayed in 90 blood samples with paired DSA and clinically indicated biopsies from 87 kidney transplant patients. Sixteen cases met criteria for active ABMR. Performance characteristics of dd-cfDNA for diagnosis of active ABMR were determined for samples with prior or current positive DSA (DSA+, n = 33). **Results.** The median level of dd-cfDNA (2.9%) in DSA+ patients with active ABMR was significantly higher than the median level (0.34%) in DSA+ patients without ABMR ($P < 0.001$). The median level of dd-cfDNA in DSA– patients was 0.29%. The positive predictive value of dd-cfDNA (at 1%) to detect active ABMR in DSA+ patients was 81%, whereas the negative predictive value was 83%. The positive predictive value for DSA+ alone was 48%. **Conclusions.** The combined use of dd-cfDNA and DSA testing may improve the noninvasive diagnosis of active ABMR in kidney transplant patients. Patients with dd-cfDNA+/ DSA+ results have a high probability of active ABMR.

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Kidney transplant patients who are sensitized or otherwise at high risk for antibody-mediated rejection (ABMR) due to previous sensitization events are monitored for donor-specific antibodies (DSA). In addition to biopsy findings, serological DSA positivity is a requirement for diagnosis of active ABMR per Banff 2013 criteria[1] and is associated with poor graft outcomes.[2] Limitations of DSA testing include high frequency of false positives in patients without rejection, variability of time between DSA emergence and manifestation of rejection,[3-7] false negatives due to sequestered antibodies in the graft or presence of non-HLA DSAs, absence of consensus criteria for clinically meaningful antigens or IgG subclasses,[8] uncertainty of clinically meaningful quantitative thresholds (mean fluorescence intensity) for clinical decisions,[9,10] fluctuating levels of DSAs, and lack of assay standardization.[11,12]

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To date, confirmation that DSA contributes to graft injury is based on histopathological evidence of active injury from invasive biopsy specimens and nonspecific, late indicators of functional impairment, such as rises in serum creatinine.

Donor-derived cell-free DNA (dd-cfDNA) provides a measure of graft injury using an accurate and reproducible, analytically and clinically validated test. The dd-cfDNA levels above 1% strongly correlate with biopsy-proven active rejection.[13] Stable kidney transplant recipients have a median of 0.21% dd-cfDNA.[14] Although T cell–mediated rejection (TCMR) is associated with lower levels of dd-cfDNA than active ABMR (1.2% for TCMR >IA vs 2.9% for active ABMR),[13] dd-cfDNA is not specifically pathognomonic for rejection, subtype of rejection, or a specific histological feature, such as glomerulopathy. This report describes how dd-cfDNA may contribute to the molecular diagnosis of ABMR. Just as other studies have demonstrated that DSA or other molecular classifiers may improve the diagnosis of ABMR beyond what is possible with histology,[1] we describe how dd-cfDNA can serve as a complement to DSA in the diagnosis of ABMR.

MATERIALS AND METHODS

The Circulating Donor-Derived Cell-Free DNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients (DART) study (ClinicalTrials.gov NCT02424227) was a prospective observational study conducted at 14 clinical sites, described in detail elsewhere.[13] The institutional review board at each site approved the study, and all the patients provided written informed consent. The study sponsor provided the statistical analysis, data management, and clinical operations coordination.

In this current report, we analyze the performance of dd-cfDNA and DSA status as independent covariates to discriminate active ABMR. The ABMR index cases included in this report were described previously.[13] In that prior report, the diagnostic impact of DSA-positive status in combination with dd-cfDNA was not analyzed. Transplant patients were enrolled and blood collected at the time of a clinically indicated kidney biopsy. Venous blood for dd-cfDNA testing (AlloSure) was collected in Streck Cell-Free DNA BCT tubes, stored at room temperature, and shipped to the Clinical Laboratories Improvements Act (CLIA) certified laboratory at CareDx, Inc., Brisbane, California. Plasma was separated and then cfDNA was extracted using the Circulating Nucleic Acid kit (Qiagen, Redwood City, CA) as described.[15] A targeted next-generation sequencing assay was used to accurately quantify dd-cfDNA without need for separate genotyping of the recipient or the donor (quantifiable range, 0.2% to 16% dd-cfDNA in total cfDNA).[15]

Information was collected on the number of, and clinical indication for, renal transplant biopsies for each patient. The onsite pathologist's renal transplant biopsy diagnostic report was used by the site investigator to guide completion of the study case report form which captured the diagnosis of rejection in accordance with criteria designated by the Banff Working Groups.[1,16] Active ABMR includes only acute/active ABMR and chronic active ABMR as defined using the nomenclature and criteria prescribed by the Banff 2013 meeting.[1] The "no ABMR" group includes all other pathology cases: partial Banff criteria for active ABMR (eg, C4d staining without evidence of rejection), TCMR, biopsy findings not classified as rejection, and those with no findings.[13] T cell–mediated rejection cases, acute or chronic active, were defined using the nomenclature and criteria of the Banff 2005 meeting (published in 2007),[16] because at the time of the design and conduct of this study, this criteria for TCMR classification was current.

Donor-specific antibodies were identified by HLA measurement methods (single-antigen bead technology and One Lambda reagents) and the criteria for DSA+ classification was determined according to existing protocols at each center. A range of thresholds were used across the centers in the study, with the most common threshold for a DSA+ being mean fluorescence intensity (MFI) > 1000. The MFI in the index cases of ABMR were most often at least 10 000. C4d positivity was also considered at some centers.

T cell–mediated rejection includes those biopsy reports that meet the Banff 2007 criteria for T cell mediated rejection types IA, IIA, IB, IIB, or III.[16] The biopsy reports that diagnosed mixed active ABMR and TCMR were grouped together with the active ABMR subgroup for purposes of the analyses.

Demographics were computed using analysis of variance for continuous variables and Fisher's test for discrete variables. The prevalence of biopsy-proven active ABMR was computed from 61 clinically indicated biopsies in the DART study (previously described[13]) with DSA+ test results. The set of visits with dd-cfDNA measurements and DSA testing results were divided into 3 groups: DSA+ with active ABMR, DSA+ with no ABMR, and DSA−, and the groups were compared using pairwise Wilcoxon Rank Sum testing. Samples were classified as positive or negative for dd-cfDNA (dd-cfDNA+ or dd-cfDNA−) based on a 1% threshold. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and area under the curve (AUC) of dd-cfDNA+ to predict active ABMR within DSA+ samples were computed. All analyses were performed with the use of R software, version 3.2.0, 64-bit, copyright 2015.

RESULTS

Patient samples, histopathology results, and other clinical data were derived from the DART study (Figure S1, http://links.lww.com/TXD/A124).[13] In this observational study, DSA-positive or -negative classification for each patient was defined by existing protocols at each center. Of the 384 patients and 1272 samples analyzed, there were 87 patients with 90 visits with a DSA result, a biopsy due to clinical suspicion, and a dd-cfDNA result. All recipients and donors were ABO blood group compatible. Of these, 33 patients (33 visits) had a current or prior DSA+, and 54 patients (57 visits) classified as DSA−. The DSA− and DSA+ groups were well matched demographically (Table S1, http://links.lww.com/TXD/A125). The age at enrollment was younger in the DSA+ than the DSA− group (47 ± 14 years vs 53 ± 12 years, respectively, $P = 0.024$).

Chronic active and acute/active ABMR were defined using the Banff 2013 criteria.[1] Sixteen samples from 16 patients were diagnosed with 1 of these ABMR types as described.[13] Of these, 5 also had a diagnosis of TCMR. The prevalence of active ABMR was estimated to be 48%; 29 of 61 samples that were DSA+ were confirmed to have active ABMR by biopsy. These included all DART visits with DSA test results and biopsy, whether or not dd-cfDNA was tested in conjunction with the biopsy (Figure S1, links.lww.com/TXD/A124).

The median level of dd-cfDNA in active ABMR (n = 16) was 2.9%. This level was significantly higher than in DSA+

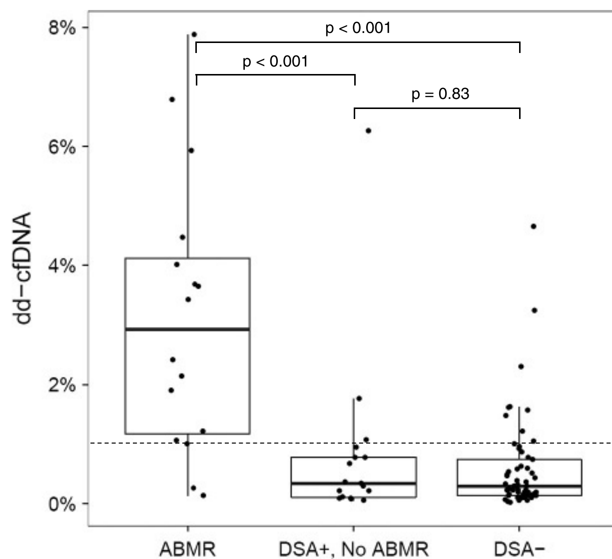

FIGURE 1. dd-cfDNA level is significantly higher in patients with active ABMR. Patients with positive DSA and a biopsy diagnosis of active ABMR had significantly elevated dd-cfDNA when compared to DSA-positive patients who are biopsy negative for ABMR (DSA+, No ABMR). dd-cfDNA for DSA− samples are not significantly different from DSA+, No ABMR. TCMR are included in the No ABMR and DSA− data. Medians are shown by the horizontal bar, 25th and 75th percentiles (interquartile range) are shown as the top and bottom of the boxes and are 2.9 [1.18-4.13] for active ABMR; 0.34 [0.11-0.78] for DSA+, No ABMR; and 0.29 [0.14-0.74] for the DSA−.

samples without biopsy-based diagnosis of ABMR (n = 17: DSA+/no ABMR, median 0.34%, $P < 0.001$, Figure 1). The 57 DSA− samples had a median dd-cfDNA of 0.29% which was similar to the DSA+/no ABMR group ($P = 0.83$). The 2 groups without ABMR (DSA+/no ABMR and DSA−) included 53 samples without rejection and 7 with biopsy-confirmed TCMR.[13]

Samples were classified as positive or negative for dd-cfDNA (dd-cfDNA+ or dd-cfDNA−) based on a 1% threshold.[13] The test performance characteristics of dd-cfDNA in diagnosis of active ABMR in DSA+ patients are summarized in Table 1. At the 1% threshold, the PPV of dd-cfDNA to detect active ABMR in DSA+ samples was 81% and the NPV was 83%. Using 2.9% dd-cfDNA, the median value for active ABMR, the PPV of dd-cfDNA to detect active ABMR in DSA+ patients was 89%. Thirteen of 16 (81%) of DSA+/dd-cfDNA+ samples had active ABMR, whereas 3 (4%) of the 74 remaining samples had active ABMR, all with dd-cfDNA at or below 1% (Table S2, http://links.lww.com/TXD/A126).

DISCUSSION

The results here suggest that dd-cfDNA may be used in conjunction with DSA status to improve the diagnosis of active ABMR in kidney transplant patients. The combined finding of dd-cfDNA+ and DSA+ resulted in an accurate noninvasive diagnosis of active ABMR. The PPV of 81% for active ABMR at 1% dd-cfDNA in DSA+ samples was much higher than either DSA alone (48%) or dd-cfDNA alone (44%).[13] The NPV of dd-cfDNA at a 1% threshold in the presence of positive DSA was 83%. The high correlation of dd-cfDNA with active ABMR in the DSA+ cohort not only provides a high NPV, but also a high PPV. The high sensitivity of dd-cfDNA to identify active ABMR in the DSA+ patients provides confidence that dd-cfDNA–negative patients are likely not experiencing active ABMR. An example patient from the DART observational study is shown in Figure 2A; 3 DSA+ results over the course of 2 years resulted in 3 invasive biopsy procedures without significant findings. These biopsy procedures might have been avoided if the patients had been managed using both DSA testing and dd-cfDNA.

Patient inconvenience and anticoagulation are known to defer biopsies, which may result in underdiagnosis of ABMR.[17] Thus, the noninvasive nature of dd-cfDNA testing may result in an earlier diagnosis of active ABMR when DSA and dd-cfDNA are both positive (Figure 2B). The refractory nature of ABMR to current treatments suggests early diagnosis is desirable.[18,19] The performance of dd-cfDNA reported here (AUC of receiver-operator characteristics curve [AUC-ROC], 86%) is superior to the performance (AUC-ROC, 66%) reported for strength of pretransplant DSA (defined by highest individual MFI value) associated with subsequent clinical ABMR.[2] Therefore, a positive dd-cfDNA in the setting of DSA positivity would provide a much higher noninvasive level of evidence to support a biopsy decision than DSA positivity alone. However, this likely depends on the strength, complement activating capacity, and IgG subclass of DSAs.[20,21] Although a biopsy may be necessary to establish the type of active rejection, dd-cfDNA measurements may provide a means for longitudinal tracking of response to treatment interventions with a frequency and quantitative accuracy not feasible with repeated biopsies or other biomarkers.

The limitations of this study include the limited number and mixed forms of active ABMR, from 6 centers. Although the centers used similar criteria to determine the DSA, pooling of their results may not be reliable due to lack of a means to standardize and calibrate the DSA assay results between centers. Baseline or longitudinal data before the biopsy was not available for these for-cause biopsies and could be important to understanding the few differences between biopsy and dd-cfDNA. There is no obvious explanation for why dd-cfDNA was not elevated in 2 of the cases where active ABMR was identified by examination of the biopsy. As more cases are accumulated, explanations may emerge, but the concordance of pathologists grading of rejection findings is not high (kappa less than 40%) so the reference standard for the diagnosis itself is not truly a "gold" standard.[22] The diagnostic criteria and classification of active ABMR has included morphologic

TABLE 1.

Performance characteristics of dd-cfDNA for diagnosis of active ABMR in DSA-positive patients

Endpoint	dd-cfDNA cutoff	Sensitivity	Specificity	AUC-ROC	PPV	NPV
Active ABMR or mixed	1%	81% (67%-100%)	82% (67%-100%)	86% (70%-98%)	81% (69%-100%)	83% (73%-100%)
Active ABMR or mixed	2.9%	50% (30%-70%)	94% (88%-100%)	86% (70%-98%)	89% (75%-100%)	68% (60%-77%)

Data presented with 95% confidence intervals. Performance characteristics shown at 1% cutoff used in previous publications, and at 2.9% for active ABMR, the median active ABMR level of dd-cfDNA. Prevalence of rejection in DSA+ samples 48%.

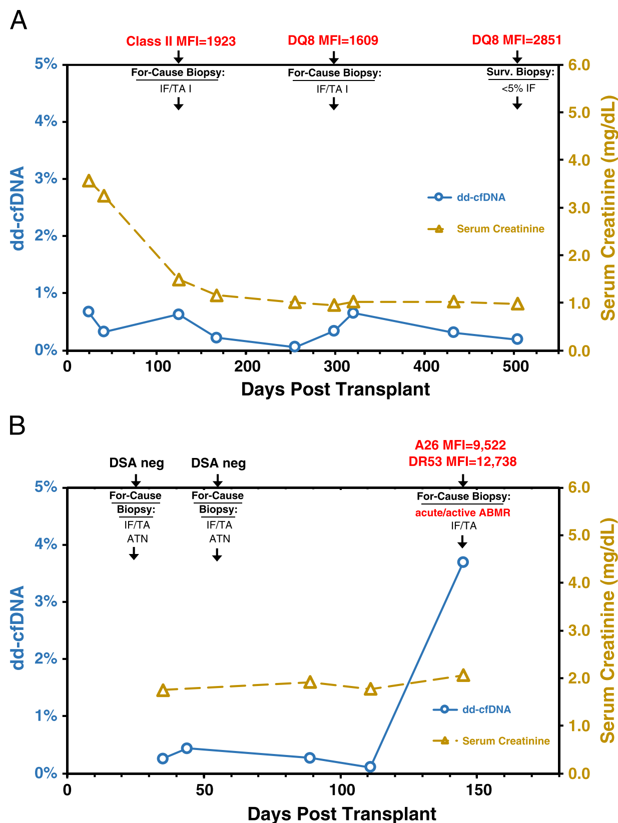

FIGURE 2. Case studies. A, This patient had 3 consecutive DSA+ results and no ABMR found in associated biopsies. Serial dd-cfDNA were all negative (below 1%). B, A significant rise in dd-cfDNA was associated with acute/active ABMR in a renal allograft recipient. dd-cfDNA level (3.7%) on day 145 posttransplantation significantly increased compared to 0.2% on day 111. Serum creatinine rose from 1.77 to 2.06. Allograft biopsies on posttransplant days 30 and 60 (see arrows) revealed mild IF/TA and focal areas of ATN. Biopsy on day 145 revealed ABMR. DSAs were neg at days 30 and 60 and pos on day 145. IF/TA, interstitial fibrosis/tubular atrophy; ATN, acute tubular necrosis; negative, neg; positive, pos.

evidence (eg, neutrophils and/or monocytes/macrophages in peritubular capillaries; glomerulitis), immunohistologic evidence (eg, diffuse C4d staining in peritubular capillaries), and serologic evidence (eg, circulating antibodies to donor HLA or other specific antidonor antibodies at the time of biopsy). These diagnostic criteria continue to evolve as evidence accrues to demonstrate the validity of new molecular biomarkers. For instance, evidence of current or recent antibody interaction with vascular endothelium may be provided by demonstrating increased expression of endothelial activation and injury transcripts or other validated gene expression markers of endothelial injury in the biopsy tissue.[1] Studies of dd-cfDNA on additional patients will benefit from detailed data collection that will allow analysis of recently defined ABMR classes, such as C4d-negative ABMR.[23] It will be of interest to extend the current study to a larger cohort of cases with different diagnoses, allowing us to reevaluate the findings in the context of recurrent or de novo glomerulonephritis.

Based on the high apparent value of the information provided by the dd-cfDNA in the presence of DSA+, performing both tests in situations that already warrant DSA testing merits consideration.[8] dd-cfDNA used together with DSA may improve the noninvasive diagnosis of active ABMR in kidney transplant patients and allow for more precise monitoring in longitudinal surveillance. These paired tests may also be useful in the longitudinal evaluation of patients after treatment for ABMR to determine the need for continued treatment, alternative treatments, or repeat biopsy. Donor-derived cell-free DNA may also be a useful noninvasive biomarker for the evaluation of ABMR response to treatment in clinical trials of new agents. Altogether, these results demonstrate that patients with dd-cfDNA+/DSA+ results have a high probability of active ABMR and patients with dd-cfDNA−/DSA+ results are unlikely to have active ABMR.

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REFERENCES

1. Haas M, Sis B, Racusen LC, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am J Transplant*. 2014;14:272–283.
2. Wehmeier C, Honger G, Cun H, et al. Donor specificity but not broadness of sensitization is associated with antibody-mediated rejection and graft loss in renal allograft recipients. *Am J Transplant*. 2017;17:2092–2102.
3. Puttarajappa C, Shapiro R, Tan HP. Antibody-mediated rejection in kidney transplantation: a review. *J Transplant*. 2012;2012:193724.
4. Wiebe C, Gibson IW, Blydt-Hansen TD, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. *Am J Transplant*. 2012;12:1157–1167.
5. Everly MJ, Rebellato LM, Haisch CE, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation*. 2013;95:410–417.
6. Wiebe C, Gibson IW, Blydt-Hansen TD, et al. Rates and determinants of progression to graft failure in kidney allograft recipients with de novo donor-specific antibody. *Am J Transplant*. 2015;15:2921–2930.
7. Schinstock CA, Cosio F, Cheungpasitporn W, et al. The value of protocol biopsies to identify patients with de novo donor-specific antibody at high risk for allograft loss. *Am J Transplant*. 2017;17:1574–1584.
8. Tait BD, Susal C, Gebel HM, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. *Transplantation*. 2013;95:19–47.
9. Tambur AR, Herrera ND, Haarberg KM, et al. Assessing antibody strength: comparison of MFI, C1q, and titer information. *Am J Transplant*. 2015;15:2421–2430.
10. Sullivan HC, Liwski RS, Bray RA, et al. The road to HLA antibody evaluation: do not rely on MFI. *Am J Transplant*. 2017;17:1455–1461.
11. Ellis TM. Interpretation of HLA single antigen bead assays. *Transplant Rev (Orlando)*. 2013;27:108–111.
12. Reed EF, Rao P, Zhang Z, et al. Comprehensive assessment and standardization of solid phase multiplex-bead arrays for the detection of antibodies to HLA-drilling down on key sources of variation. *Am J Transplant*. 2013;13:3050–3051.
13. Bloom RD, Bromberg JS, Poggio ED, et al. Cell-free DNA and active rejection in kidney allografts. *J Am Soc Nephrol*. 2017;28:2221–2232.
14. Bromberg JS, Brennan DC, Poggio ED, et al. Biological variation of donor-derived cell-free DNA in renal transplant recipients: clinical implications. *J Appl Lab Med*. 2017;2:309–321.
15. Grskovic M, Hiller DJ, Eubank LA, 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.
16. Solez K, Colvin RB, Racusen LC, et al. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). *Am J Transplant*. 2007;7:518–526.
17. Gloor JM, Cohen AJ, Lager DJ, et al. Subclinical rejection in tacrolimus-treated renal transplant recipients. *Transplantation*. 2002;73:1965–1967.
18. Valenzuela NM, Reed EF. Antibody-mediated rejection across solid organ transplants: manifestations, mechanisms, and therapies. *J Clin Invest*. 2017;127:2492–2504.

19. Malvezzi P, Rostaing L. Renal transplantation in 2016: novel approaches to improve recipient and allograft outcomes. *Nat Rev Nephrol*. 2017;13:73–74.
20. Lefaucheur C, Viglietti D, Bentlejewski C, et al. IgG donor-specific anti-human HLA antibody subclasses and kidney allograft antibody-mediated injury. *J Am Soc Nephrol*. 2016;27:293–304.
21. Loupy A, Lefaucheur C, Vernerey D, et al. Complement-binding anti-HLA antibodies and kidney-allograft survival. *N Engl J Med*. 2013;369: 1215–1226.
22. Reeve J, Sellares J, Mengel M, et al. Molecular diagnosis of T cell-mediated rejection in human kidney transplant biopsies. *Am J Transplant*. 2013;13:645–655.
23. Haas M, Loupy A, Lefaucheur C, et al. The Banff 2017 Kidney Meeting Report: revised diagnostic criteria for chronic active T cell–mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am J Transplant*. 2018;18: 293–307.