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Donor-derived Cell-free DNA: Advancing a Novel Assay to New Heights in Renal Transplantation

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Abstract. Despite advances in transplant immunosuppression, long-term renal allograft outcomes remain suboptimal because of the occurrence of rejection, recurrent disease, and interstitial fibrosis with tubular atrophy. This is largely due to limitations in our understanding of allogeneic processes coupled with inadequate surveillance strategies. The concept of donor-derived cell-free DNA as a signal of allograft stress has therefore rapidly been adopted as a noninvasive monitoring tool. Refining it for effective clinical use, however, remains an ongoing effort. Furthermore, its potential to unravel new insights in alloimmunity through novel molecular techniques is yet to be realized. This review herein summarizes current knowledge and active endeavors to optimize cell-free DNA-based diagnostic techniques for clinical use in kidney transplantation. In addition, the integration of DNA methylation and microRNA may unveil new epigenetic signatures of allograft health and is also explored in this report. Directing research initiatives toward these aspirations will not only improve diagnostic precision but may foster new paradigms in transplant immunobiology.

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INTRODUCTION

Kidney transplantation (KT) affords individuals with end-stage renal disease superior survival and quality of life over conventional dialytic therapies.¹ Sustaining this survival advantage relies on effective allograft surveillance that consists of serial laboratory testing as well as percutaneous biopsy.² Although the latter is regarded as the gold standard in defining the pathogenesis for allograft injury such as rejection, it has limitations such as sampling error, cost as well as carrying risks of bleeding and organ injury.³ This has led to the pursuit of non-invasive strategies including detection of donor-derived cell-free DNA (dd-cfDNA) as a marker of allogeneic injury. Since becoming Medicare reimbursable in the

United States since October 2017, questions have arisen regarding the practical utility of dd-cfDNA in renal allograft management.⁴ This review discusses the role and limitations of this technology as well as offering insights into current challenges and future applications.

Causes of Allograft Injury and Current Surveillance Strategies

There has been progress in improving renal allograft survival in the first-year posttransplantation; however, efforts are still needed with respect to long-term graft outcomes.^{5,6} Nonsurgical causes of allograft failure are likely dependent on population characteristics, immunosuppression practice patterns along with allograft surveillance protocols.⁷ In a prospective cohort analysis of some 315 North American renal transplant recipients undergoing indication biopsies, allograft failure occurred in 54 patients because of rejection in (64%), glomerulonephritis (18%), polyomavirus nephropathy (7%), and intercurrent events (11%).⁸ Among these cases, antibody-mediated rejection (AMR) was identified universally in those with allograft loss from rejection, highlighting the significance of this allogeneic process in terms of clinical outcome. Interestingly, pure acute T cell-mediated rejection (ACR) was not seen in those with failed grafts in this study population. In a separate large UK cohort, aside from allograft failures due to death with functioning graft and allogeneic injury, recurrent primary disease and infection were also noted as potential drivers of allograft failure.⁹ Despite these advances in detecting and defining renal allograft injury, in a significant number of cases the causes of graft loss is not clearly identified. This has previously been referred to as a chronic allograft nephropathy and is now

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termed interstitial fibrosis and tubular atrophy (IFTA) following the eighth Banff conference in 2005.^{10,11} Hypothesized mechanisms for this entity may include ischemia-reperfusion injury (IRI), subclinical rejection, and calcineurin-inhibitor nephrotoxicity.¹²

Although these studies yield sobering results, it must be acknowledged that they reflect a surveillance strategy contingent on percutaneous allograft biopsy. Despite its risks, this approach using protocol biopsies was widely adopted upon recognition that early detection and treatment of subclinical rejection may be beneficial.^{13,14} Inflammation in areas of interstitial fibrosis was also subsequently demonstrated to predict allograft outcomes further supporting the role of surveillance biopsies in routine clinical practice.¹⁵ The cost and cumbersome nature of surveillance biopsies, however, remains a mitigating factor for many transplant programs, and has been cited at \$3931 holistically per procedure according to the CMS Physician Fee Schedule from 2015.¹⁶⁻¹⁸

Principles of Cell-free DNA

Origins and Evolution in Clinical Care

Circulating cell-free DNA was originally observed by Mandel and Matais in 1948; however, its significance would not be realized till several decades later.¹⁹ Most plasma DNA is histone bound with a short half-life (10–15 min) and belongs to the Alu repeat family that may originate from apoptosis or via direct cellular secretion.²⁰⁻²² Tissue necrosis is not believed to be a major source given that tumor derived cell-free DNA has been observed to decrease with cytotoxic therapy.²³ Its presence is known to be associated with various disease states including autoimmunity, infections, malignancy, ischemia, and trauma.²⁴⁻²⁸ Cell-free DNA has thus found clinical value in oncology, prenatal care, and solid organ transplantation.²⁹⁻³¹ The latter depends on delineating donor from host-circulating DNA to serve as a signal of allograft stress (refer to Figure 1).³² In 1998, Lo et al³² reported that donor-derived DNA is present in the plasma of kidney and liver transplant recipients. They envisioned that donor DNA might be used as a diagnostic tool for detecting transplant rejection, which became a reality with the advancement in molecular techniques and throughput sequencing.

Kinetics of Cell-free DNA

In kidney transplant recipients, dd-cfDNA levels of <1% of total cfDNA appear to reflect the absence of active rejection.³³ In the presence of rejection, donor cell injury results in its release

into the blood stream. The majority of this cfDNA is likely a product of active apoptosis as fragmented DNA gets disseminated into the circulation.³⁴ The half-life and the rate of clearance of cfDNA from blood remain unclear. A combination of nuclease degradation, clearance by the kidney, and uptake by the liver and spleen is likely to play a role.³⁵ Thus, its half-life is likely to be prolonged in the context of impaired kidney function.

Cell-free DNA and the Immune Response

Aside from its prospects as a diagnostic tool, characterizing dd-cfDNA as a potential damage-associated molecular pattern (DAMP) raises some interesting possibilities. DAMPs encompass a range of endogenous biomolecules that are released upon cellular damage, which can activate the innate immune system via interaction with pattern recognition receptors such as Toll-like receptors.³⁶ Indeed, the capacity of DNA to serve as a DAMP via interaction with TLR-9 to drive activation of the innate immune system has been reported.³⁷ This may hold relevance in solid organ transplantation since the role of the innate immune response in allograft tolerance and rejection has been described.³⁸ For instance, in a series of skin transplant experiments using a mouse model, Goldstein et al³⁹ demonstrated the role of TLR signaling via MyD88 (a TLR signal adapter protein) in mediating allograft rejection. The significance of this pathway was similarly replicated in a murine renal transplant model.⁴⁰ There is also evidence that TLR signaling via MyD88 is important in dendritic cell function and T-helper 1 cellular responses as part of the allogeneic process.⁴¹ Oetting et al⁴² have described the significance of donor polymorphisms in TLR4 in graft outcomes following liver transplantation, extending this concept to human subjects. Furthermore, DAMPs released during renal injury may also activate inflammasomes, which are cytoplasmic protein complexes triggered in infectious or sterile injuries.⁴³

Technical Approaches and Currently Available Assays

There are various approaches for detecting dd-cfDNA ranging from copy number variation (CNV), molecular assays targeting the Y chromosome in female recipients of allografts from male donors, to more sophisticated genome transplant dynamic (GTD) strategies, which conventionally requires donor and recipient genomic data (2 genome-approach). The role of gene copy number in genetic disorders has been described by Inoue and Lupski and applying this concept solely to distinguish donor from host DNA is the basis of CNV.⁴⁴ CNV is a DNA segment of 1 kb or larger that is present at a variable

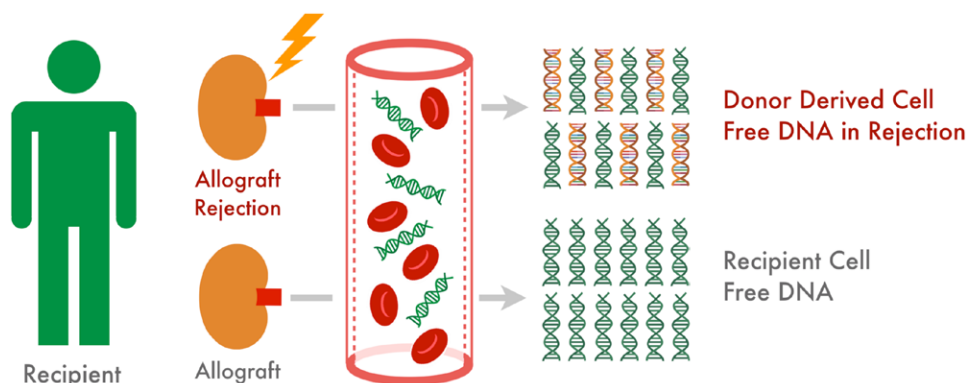


FIGURE 1. Concept of donor-derived cell-free DNA as a marker of allograft injury.

copy number in comparison with a reference genome. Organ transplantation is essentially genomic transplantation and the distinctive graft and recipient genotype single-nucleotide polymorphisms (SNPs) can be used to barcode donor DNA circulating in recipient serum. GTD utilizes SNPs distributed across the genome to discriminate donor from recipient DNA. It was first demonstrated as proof of concept in a retrospective analysis of heart transplant recipients in 2011.⁴⁵ GTD methodology has subsequently been clinically validated in solid organ transplantation.⁴⁶ A 1-genome strategy for quantifying dd-cfDNA in the absence of donor genotype through mathematical modeling was recently described by Sharon et al.⁴⁷

At the time of publication, there are 3 dd-cfDNA assays widely available for clinical use. The Allosure Test (CareDx, Inc) exploits a panel of 266 SNPs known to be different among individuals based on work by Pakstis et al.⁴⁸ Another method utilized by Sigdel et al which is based on work from cell-free DNA analytics involving massively multiplexed-PCR targeting 13 392 SNPs and is currently available as Prospera through Natera.^{49,50} A third assay—Viracor TRAC is also available through Eurofins Clinical Diagnostics and was recently combined with TruGraf (Eurofins—Transplant Genomics Inc.), a DNA microarray-based gene expression test. Table 1 compares widely used dd-cfDNA assays based on available information.^{33,46,51,52}

Defining Cell-free DNA Using One-genome Strategy

Although an exhaustive description of 1-genome GTD methods are beyond the scope of this review, the basic steps in the detection of dd-cfDNA (as adopted in currently available assays) may be summarized.

Primer Design

The single-genome GTD approach exploits interindividual genomic variations as the basis of delineating donor from recipient DNA. Outside of the major histocompatibility complex region on the short arm of chromosome 6, the genome is highly conserved across the human population with approximately 99.9% similarity between individuals in their DNA sequence.⁵³ Characterization of nonmajor histocompatibility complex genomic variations (for use in areas such as forensic genetics) has elucidated 3 types of polymorphisms: Microsatellites or short tandem repeats, variable number

tandem repeats, and SNPs.⁵⁴ Under this concept, primers may be designed according to a curated panel of SNPs that facilitates interindividual genomic distinction. Factors deemed important in SNP selection include minor allele frequency, polymerase error rate, heterozygosity, linkage disequilibrium, and fixation index, and the reader is directed to the review by Dengu for in-depth account of these considerations.⁵⁵ For instance, 85 SNPs identified by Pakstis et al and an additional 181 SNPs incorporated by Grskovic et al form the basis of the Allosure assay made available through CareDx, Inc.^{46,48} In contrast, Prospera utilizes allelic frequency at 132 926 SNPs through prior work using SNP multiplexed polymerase chain reaction in the prenatal and oncology contexts.^{56,57}

Cell-free DNA Amplification

Targeted amplification to sequence predefined SNPs of interest (as well described in the prenatal diagnostics literature), contrasts with quantitative whole genome sequencing.⁵⁷ This entails a process of preamplification and amplification using multiplex PCR reactions.⁴⁶ The generated PCR amplicons are then barcoded for next generation sequencing (NGS).⁵¹ The details of these steps obviously vary according to individual proprietary protocols along with their SNP selections as stated above.

Next Generation Sequencing and Data Analysis

PCR amplicons are sequenced using a next generation sequencer such as the Illumina MiSeq instrument.^{46,51} Customized bioanalytic pipelines are used to quality check and process raw data to yield allele counts, which may in turn be inputted in a computational model and was described in a proof of concept report by Sharon et al.⁴⁷ This involves aligning amplicon data to the reference hg19 genome using an aligning tool such as Bowtie 2 followed by filtering unmapped or nonuniquely mapped reads using a program such as SamTools.^{47,58,59} This pipeline also incorporates barcode trimming, eliminating genotype-biased mapping, PCR duplicate removal, computing chromosomal coverage, and allele counting.^{46,47,60} The model henceforth gives an estimate of dd-cfDNA (as a percentage) based on the allele counts generated among other considerations.

Clinical Utility in Renal Transplantation: Current Evidence and Ongoing Efforts

In an endeavor to elucidate a role for dd-cfDNA in renal allograft management, numerous clinical studies have been undertaken to date (refer to Table 2).^{33,61-86} Observational data suggest that KT recipients with stable allograft function exhibit a wide range of dd-cfDNA levels immediately post-surgery with a median percentage of 10.02% at postoperative day 1 that decreases to a mean of 0.46% after 10 days.⁸⁷ Bromberg et al elucidated the biological variation and clinical reference intervals of dd-cfDNA in kidney transplant recipients with stable allograft function and determined that a cut-off of 1.0% dd-cfDNA delineated the 96th percentile of test results.⁸⁸ Most centers including ours, therefore postpone testing until 10–14 days posttransplantation given that IRI likely predominates till that point.

Acute Cellular Rejection

The circulating donor-derived cell-free DNA in blood for diagnosing acute rejection in kidney transplant recipients

TABLE 1.
Comparison of allosure and prospera cell-free DNA assays^{33,46,51,52}

	Allosure	Prospera
Number of SNPs	266	13 962
Limit of detection	0.16% (Grskovic et al)	0.15% (related donors) 0.23% (unrelated donors) (Altug et al)
Limit of quantification	0.2% (Grskovic et al)	0.15% (related donors) 0.23% (unrelated donors) (Altug et al)
Sensitivity	0.45 (Melancon) 0.59 (Bloom et al)	0.55 (Melancon) 0.887 (Sigdel et al)
Specificity	0.85 (Melancon) 0.85 (Bloom et al)	0.69 (Melancon) 0.726 (Sigdel et al)
Coefficient of variation	7.7% (dd-cfDNA < 2%) 4.5% (dd-cfDNA ≥ 2%) (Grskovic et al)	4.29% (Altug et al)

dd-cfDNA, donor-derived cell-free DNA.

TABLE 2.**Summary of studies of cell-free DNA in kidney transplantation.**^{33,61-86}

Name	Study type	Organ(s)	Patients (N)	Sample type	dd-cfDNA method
Shen et al	Prospective multicenter	Kidney	28	Plasma	Informative SNP panel
Stites et al	Prospective multicenter	Kidney	79	Plasma	Informative SNP panel
Zhang et al	Prospective multicenter	Kidney	37	Plasma	Informative SNP panel
Gieles et al	Prospective multicenter	Kidney	107	Plasma	Informative SNP panel
Oellerich et al	Prospective single center	Kidney	189	Plasma	Informative SNP panel
Bloom et al	Prospective multicenter	Kidney	384	Plasma	Informative SNP panel
Brubaker et al	Prospective single center	Kidney	27	Plasma	Informative SNP panel
Giellis et al	Prospective multicenter	Kidney	11	Plasma	Informative SNP panel
Goh et al	Prospective multicenter	Kidney	10	Plasma	INDEL allele polymorphism
Huang et al	Prospective single center	Kidney	25	Plasma	Informative SNP panel
Jackson et al	Prospective single center	Kidney	8	Plasma	Informative SNP panel
Kollmann et al	Retrospective single center	Kidney	20	Plasma	INDEL allele polymorphism
Lada et al	Case report single center	Kidney	1	Plasma	NR
Lee et al	Prospective single center	Kidney	34	Plasma and urine	Informative SNP panel
Moreira et al	Prospective single center	Kidney	17	Plasma and urine	Sex mismatch (TSPY gene)
Sigdel et al	Retrospective single center	Kidney	21	Urine	Sex mismatch (SRY gene)
Sigdel et al	Retrospective single center	Kidney	NR	Plasma and Urine	Custom primer sets
Stolz et al	Prospective single center	Kidney	13	Plasma	Informative SNP panel
Whitlam et al	Prospective single center	Kidney	31	Plasma	Deletion copy variation
Zhang et al	Prospective single center	Kidney	61	Urine	Sex mismatch (SRY gene)
Zhang et al	Retrospective single center	Kidney	37	Plasma	Cell-free -Alu DNA
Zhong et al	Prospective single center	Kidney	25	Urine	Sex mismatch (SRY gene)
Gadi et al	Retrospective single center	SPK	42	Plasma	HLA Mismatches
Beck et al	Retrospective single center	Kidney/liver/heart	34	Plasma	Informative SNP panel
Beck et al	Retrospective single center	Kidney/heart	NR	Plasma	Informative SNP panel
Woodward et al	Retrospective multicenter	Kidney/heart	114	Plasma	Sex Mismatch-TSPY1 gene
Mieczkowski et al	Retrospective single center	Kidney/liver	16	Plasma	Informative SNP panel

dd-cfDNA, donor-derived cell-free DNA; SNP, single-nucleotide polymorphism.

(DART) study, which used the aforementioned Allosure assay, was a prospective observational undertaking of plasma dd-cfDNA levels among 102 KT recipients with 107 surveillance and for-cause biopsy samples.³³ Of note were higher dd-cfDNA levels detected in cases with biopsy-proven ACR 1B (median 1.2%), whereas biopsies with ACR 1A (median 0.3%) acute tubular necrosis (ATN) and calcineurin-inhibitor toxicity had comparatively lower levels of dd-cfDNA. However, in another study dd-cfDNA levels measured via the Allosure test did not discriminate adult patients with ACR from those without rejection, as the AUC for ACR was 0.42 (95% CI, 0.17-0.66) in this particular analysis.⁸⁹ However, in a group of 13 pediatric patients, 2 cases of ACR exhibited dd-cfDNA levels of >2% and were statistically higher than those without rejection.⁷⁷ A recent prospective multicenter analysis found dd-cfDNA levels >0.5% to be predictive of adverse clinical outcomes among individuals with borderline rejection and ACR 1A on allograft biopsy.⁹⁰ If corroborated in larger studies, this may be clinically relevant since borderline rejection is a heterogeneous diagnostic category with variable prognostic outcomes.⁹¹ In contrast to the DART study, in which relatively lower dd-cfDNA fractions were found in ACR compared with AMR using the Allosure test, the use of multiplexed-PCR with 13 392 SNP targets among 300 samples from 217 biopsy matched cases demonstrated higher levels among all types of rejection with 2.2%, 2.7%, 0.6%, 0.7%, and 0.4% in AMR, ACR, borderline changes, cases with “other injury,” and stable renal allograft biopsies, respectively.⁵⁰ This assay was deemed applicable for clinical use in KT patients based on detection

limits, linearity, accuracy, and precision within a validation cohort in a study funded and conducted trial by Natera, Inc.⁵¹

Antibody-mediated Rejection

Among the DART study population, higher levels of dd-cfDNA were also seen in those with biopsy-proven AMR (median 2.9%), which based on cutoff of 1% translated to an area under the receiver operating characteristic curve (AUC) of 0.74 (95% CI, 0.61-0.86) and positive and negative predictive values for active rejection of 61% and 84%, respectively.³³ In another reanalysis of DART study samples by Weir et al, cell-free DNA was deemed to be more accurate than changes in serum creatinine for detecting rejection.⁹² As far as AMR is concerned, Jordan et al's findings were consistent with the observations from the DART study in that dd-cfDNA of >1% derived through the same means was associated with the presence of donor-specific antibodies (DSAs), and that threshold conferred a positive and negative predictive value for AMR of 81% and 83%, respectively.⁹³ Furthermore, incorporating levels of dd-cfDNA with levels of donor-specific antibody may also delineate ACR from AMR.^{33,93} Finally, dd-cfDNA has been associated with de novo donor-specific antibodies raising the possibility that donor-derived nucleic acids may independently elicit a humoral immune response.⁹⁴ Such a causal relationship if present may construe dd-cfDNA a heralding signal of impending antibody-mediated injury.

Other Forms of Allograft Injury

It is clear that (mechanistically) dd-cfDNA release is not limited exclusively to allograft rejection. Nonalloimmune

processes including IFTA, ATN, recurrent glomerulonephritis, and other disease states may also be associated with elevations in dd-cfDNA levels and are reviewed here.

Given its elusive causes, targeting therapies for IFTA is challenging, which can render the risk of allograft biopsy clinically unjustifiable. Nevertheless, it is understood mechanistically as a form of downstream cellular injury, which could conceivably lead to release of cfDNA. Although no dedicated study has been undertaken, a recent series comparing dd-cfDNA levels between 12 stable repeat- and 202 single-KT recipients from the DART cohort showed higher levels among those with a prior transplant.⁹⁵ In a separate study, there was also a trend toward higher dd-cfDNA levels among subjects with IFTA on biopsies; however, this was not statistically significant and it was also in the setting of AMR.⁸⁶ Taken together, it may be inferred that IFTA may be associated with somewhat higher dd-cfDNA levels but to what extent has yet to be defined.

As is the case for IFTA, there are no published data on dd-cfDNA levels specifically in the setting of ATN. This is not surprising since biopsy is rarely undertaken when suspicion for ischemic tubular injury is favored over rejection. In a study of total rather than donor-derived cfDNA, Moreira et al reported significantly higher levels in acute rejection and drug toxicity than ATN.⁶³ Similarly, results of the DART study also suggested more significant dd-cfDNA release in the context of allogeneic injury than in ATN.³³ Considering that elevated levels are seen perioperatively when IRI is most profound, dd-cfDNA is likely reflective of the extent and not solely the cause of allograft stress.

Recurrent disease remains an important source of death-censored graft loss with incidence varying depending on pathogenesis, population characteristics, and follow-up time periods.^{96,97} There is currently a paucity of data on dd-cfDNA levels in this setting; however, host-derived circulating DNA has been reported in systemic lupus erythematosus, progressive diabetic kidney disease, and urinary mitochondrial DNA has also been described in IgA nephropathy.⁹⁸⁻¹⁰⁰ It should be noted that cell-free DNA may be extruded during the inflammatory process of neutrophil extracellular traps formation.¹⁰¹ NETosis involves the deployment of extracellular chromatin, which is speculated to entangle microbes as host-defense mechanism, and is seen in a number of autoimmune conditions.^{102,103} It is therefore plausible, although unproven that inflammatory glomerular diseases may be associated with higher cell-free DNA levels compared with noninflammatory conditions. Whether donor cells exhibit similar DNA release in recurrent disease states also needs to be investigated.

Although appraisals of these 2 available tests clearly show higher fractions of dd-cfDNA in cases of rejection compared with other allograft states, there has been no dedicated study to date looking at levels in the context of allograft infections, urologic, or hemodynamic-mediated insults. Nevertheless in a subsequent analysis of 11 individuals among the DART cohort with BK viremia, 7 had viral titers that correlated with elevated dd-cfDNA levels and histopathology findings; however, the sample size was inadequate to draw further conclusions regarding the detection of BK nephritis.¹⁰⁴ In other forms of organ stress, it may also be speculated that greater dd-cfDNA is released as a result of cellular injury. Anecdotally, a markedly elevated level has been seen in a case of Page kidney following traumatic allograft biopsy at our center in a 28-year-old male, which normalized postsurgical intervention

and with recovery in the acute kidney injury. To what extent dd-cfDNA levels correlate with specific disease states has yet to be determined.

Utility in Multiorgan Transplantation

Other questions regarding the usability of dd-cfDNA include interpretation in multiorgan transplant recipients. The aforementioned comparison of stable single- and repeat-KT recipients found that dd-cfDNA levels remained <1% despite the higher levels seen among the latter, and that levels were further elevated during rejection.⁹⁵ Based on this work and the established threshold for dd-cfDNA levels, it is reasonable to utilize this assay in multiorgan transplant recipients. Nonetheless, it is important to recognize, however, that the currently available assays cannot discern which allograft is injured in the setting of an elevated dd-cfDNA level.

Current Challenges

Although cell-free DNA-based diagnostic tests have rapidly been accepted and implemented in the care of transplant patients, there are several issues and unanswered questions that need to be addressed.

Quantification of Donor-derived Cell-free DNA

The use of NGS-based platforms and indeed the method of dd-cfDNA quantification in itself has been questioned. Whitlam et al have pointed out potential bias arising from DNA preamplification along with advantages of CNV in absolute quantification of dd-cfDNA (as opposed to a percentage), which also overcomes changes in circulating recipient-derived cfDNA.¹⁰⁵ The latter may be particularly important during physiologic stress in which the recipient's leukocyte-derived cfDNA becomes the predominant source of total cfDNA, undermining the accuracy of a percentage.^{85,106} Using CNV and droplet digital PCR, Whitlam et al found absolute graft derived cf to be more specific for AMR, whereas fractionated estimates had more diagnostic utility for chronic active antibody-mediated rejection (CAAMR) and ACR.¹⁰⁵ Furthermore in a recent blinded prospective study, absolute dd-cfDNA demonstrated superiority in discriminating the presence versus absence of rejection or other forms of graft injury compared with a fractionated metric.⁸⁵ Although the findings of both reports suggest value in absolute quantification of dd-cfDNA, they still retain a role for fractionated dd-cfDNA in clinical practice. Further work is therefore necessary to refine this assay in the context of the highly dynamic host-derived cfDNA milieu.

Improving Predictability

The superiority of dd-cfDNA over serum creatinine in detecting rejection has been demonstrated.⁹² However, defining a threshold that can prompt therapy *in advance* of histologic changes continues to be a challenge given the risk of false positives. Based on work from Bromberg et al, normal biological variation of up to 61% may occur in serial laboratory values but the role of trending levels to predict rejection is not known.⁸⁸ Adopting measures of DNA methylation and microRNA may help overcome these issues and are discussed below.

Role in Monitoring Therapy

In oncology cfDNA has been used for real-time molecular monitoring of treatment, detection of recurrence, and tracking

resistance. If such “liquid biopsy” could be used to complement traditional markers to guide therapy, it could be transformative in transplant medicine. Although there is currently limited experience with cell-free DNA as a tool to monitor response to therapy, at time of publication, we note a registered trial examining the use of cfDNA to characterize therapeutic response with tocilizumab in CAAMR.¹⁰⁷ This study, which is due for completion in December 2020, will define changes in dd-cfDNA in conjunction with histopathologic changes in response to monthly therapy with tocilizumab for CAAMR.

Future Directions: Enhancing Diagnostic Precision and Wider Applications

There is an ongoing quest to improve the diagnostic accuracy of existing cfDNA platforms through incorporation of new and novel molecular markers such as DNA methylation pattern and microRNAs. This may carry important implications with respect to renal allograft assessment, monitoring and transplant therapeutics. Furthermore, there is opportunity to expand the use of cfDNA (beyond that which is solely donor-derived) to detect infectious states that transplant recipients are typically predisposed to.

Integration of DNA Methylation

Although the mere presence of circulating dd-cfDNA is evidently significant, its characteristics and the role of host-derived cfDNA are also points of interest. In particular, the concept of *epigenetic phenomenon* as the interface between the genome and the environment potentially represents another biomarker of renal disease states.¹⁰⁸ This essentially reflects the interplay between epigenetic modification including DNA methylation and gene expression in both physiological and pathophysiological conditions.¹⁰⁹ DNA methylation is generally associated with gene silencing, whereas hypomethylation is associated with permissive gene expression. Indeed, there is evidence that genome-wide epigenetic modification are associated with decline in native renal function in those with established chronic kidney disease.¹¹⁰ Whether these observations hold true with respect to allograft health and function has yet to be explored. In addition, epigenetic factors have been described in the maturation and function of memory T cells, T-reg cells, B cells, and NK cells.¹¹¹⁻¹¹³ Defining these epigenetic “signatures” may offer new insights into allogeneic processes and may foster novel diagnostic models of rejection.

Circulating microRNA

While this review primarily targets cfDNA, the prospect of enhancing diagnostic specificity through current insights in epigenetics warrants a brief review of such factors. Essentially gene expression may be influenced by noncoding RNA such as small interfering RNAs and microRNAs (miRNAs), which are linked with epigenetic processes such as DNA methylation and histone modifications.¹¹⁴ Extracellular miRNA is known to be present in plasma and serum, and its role in intercellular signaling is also recognized.^{115,116} In fact, altered expression of a specific miRNA is also believed to be associated with renal allograft rejection possibly through modifying the expression of certain genes in regulatory T cells.¹¹⁷ Thus, whether integration of miRNA signature to dd-cfDNA will improve the diagnostic accuracy needs to be studied.

DAMP Signaling and Therapeutic Potential of Cell-free DNA

As alluded to above, characterizing dd-cfDNA as a candidate DAMP molecule may afford new insights in transplant immunology. The role of such mediators in sterile inflammation in autoimmunity, malignancy, and cardiovascular disease is well recognized.¹¹⁸⁻¹²⁰ Moreover, the concept of DAMPs as a therapeutic target in a cancer model has also been proposed.¹²¹ Applying this theory using dd-cfDNA may therefore cultivate new knowledge in mechanisms and treatment of allograft rejection.

Cell-free DNA for Microbial Diagnostics Posttransplantation

Fragments of genomic DNA from pathogens causing infection at various locations in the body are found in purified plasma cfDNA. Microbial cfDNA can be detected by NGS and could identify the pathogens accurately.²⁵ The Karius Test relies on sequencing of microbial cfDNA circulating in plasma to identify over various pathogens, including bacteria, viruses, and fungi.¹²² Generally, high concentrations of microbial cfDNA have been associated with true infections, whereas low concentrations could still be due to true infections versus commensal, colonizing, or contaminant states of unknown clinical significance. This is particularly relevant in the renal transplant recipient who is prone to opportunistic infections and colonization. The Karius assay has shown sensitivity in detecting CMV and BK virus in stem-cell transplant recipients, but it has yet to be confirmed if this is generalizable in renal transplantation.¹²³ Given that these viruses can reflect overimmunosuppression but are also associated with allograft rejection makes clinical management particularly difficult.^{124,125} Integrating cfDNA of donor and microbial origin may offer an opportunity to navigate this challenging dilemma and warrants further investigation.

CONCLUSION

The adoption of noninvasive methods in renal allograft monitoring is evolving rapidly and the transplant community must recognize the strengths and caveats of these novel methods. In doing so, the field must also integrate other insights such as epigenetic analyses to further advance the diagnostic potential of cfDNA. This is particularly important given the cost, inconvenience, and limitations of percutaneous allograft biopsies. A practical question raised in this review includes the optimal surveillance interval of this assay, which has yet to be defined, particularly given the short half-life of cell-free DNA. Coupled with this issue is the assay’s usability following therapy, which will hopefully become better understood through the ongoing clinical trial described above. Another challenging scenario concerns the multiorgan transplant recipient or allograft monitoring in the setting of pregnancy where making finer-genomic delineations is necessary.

Although overcoming practical issues is important, ultimately ensuring that dd-cfDNA assay translates into overall benefit in patient and allograft outcomes is paramount. To date, there is no published report on how such testing impacts allograft survival or whether there is a cost-benefit compared with percutaneous tissue sampling. It must also be acknowledged that false positive results may lead to unnecessary allograft biopsies and paradoxically expose patients to

discomfort or harm. Another important consideration will be the influence on immunosuppressive management decisions. Long-term risks related to overimmunosuppression in the form of infections, metabolic syndrome, and malignancy are real and balancing these issues in any allograft surveillance strategy is essential. In summary, we regard dd-cfDNA as a complex yet promising biomarker of allograft health and more work will be needed to optimize it for routine use in the renal transplant population.

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