Myriad Applications of Circulating Cell-Free DNA in Precision Organ Transplant Monitoring

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Abstract

Solid organ transplantation remains the preferred treatment for many end-stage organ diseases, but complications due to acute rejection and infection occur frequently and undermine its long-term benefits. Monitoring of the health of the allograft is therefore a critically important component of post-transplant therapy. Here, we review several emerging applications of circulating cell-free DNA (cfDNA) in the post-transplant monitoring of rejection, infection,

and immunosuppression. We further discuss the cellular origins and salient biophysical properties of cfDNA. A property of cfDNA that has been prominent since its discovery in the late 1940s is its ability to yield surprises. We review recent insights into the epigenetic features of cfDNA that yet again provide novel opportunities for transplant monitoring.

Keywords: cell-free DNA; patient monitoring; diagnosis; infection; rejection

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The discovery of circulating cell-free DNA (cfDNA) predates the discovery of the double-helix structure of DNA: in 1947, Mandel and Metais (1) reported the first observation of cfDNA in the blood circulation and pointed at notable differences in the quantity of cfDNA associated with pregnancy and malignancies, already hinting at a bright future in prenatal testing and cancer monitoring. It would take another five decades before cfDNA would become relevant in the context of solid-organ transplantation, when, in 1998, Lo and colleagues (2) reported the presence of donor-derived DNA in the plasma of kidney and liver transplant recipients. In this seminal paper, Lo and colleagues hypothesized that donor DNA might be used as a marker of transplant rejection. With the advent of sophisticated molecular analytic techniques in the early 2000s, in particular high-throughput sequencing,

the understanding of the phenomenon of cfDNA was greatly improved, and a wide range of applications has been explored (3). Here, we review recent advances in solid-organ transplantation (Figure 1), where cfDNA is rapidly filling a critical medical need for more informative, noninvasive assays of acute rejection, infection, and immunosuppression.

Donor DNA as a Marker of Allograft Rejection

For female recipients of a male graft, it is relatively straightforward to identify and enumerate donor-specific cfDNA (cfDNA) through molecular assays targeting Y-chromosome DNA. Using this principle, Lui and colleagues (4) reported the presence of cfDNA in the plasma of heart, liver, and kidney transplant patients, whereas García Moreira

and colleagues (5) and Gadi and colleagues (6) provided early evidence that donor DNA is a marker of transplant injury. In 2011, a multidisciplinary group at Stanford University led by Stephen Quake introduced a method called “genome transplant dynamics” (GTD) that enables quantification of donor-specific DNA, regardless of the sex of the transplant donor or recipient (7). GTD takes advantage of single-nucleotide polymorphisms (SNPs) distributed across the genome to discriminate donor and recipient DNA molecules. This concept was first demonstrated in a retrospective study in heart transplantation, where increased levels of donor-derived DNA were shown to correlate with acute cellular rejection events, as determined by endomyocardial biopsy (7).

The utility of GTD in monitoring acute rejection was subsequently tested in two prospective studies performed at Stanford in

Figure 1. Cell-free DNA provides an information-rich window into human physiology with expanding applications in solid-organ transplant monitoring. Diagram of the procedure to collect, sequence, and identify donor-specific cell-free DNA (*top panel*; modified with permission from Reference 9). Unbiased sequencing can also be applied to observe and monitor shifts in the virome following transplantation (*bottom panel*; modified with permission from Reference 18).

heart and lung transplantation (8, 9). In this work, pretransplant SNP genotyping and post-transplant shotgun sequencing of cfDNA were used to enumerate donor- and recipient-specific SNPs. A total of 65 heart transplant recipients and 51 lung transplant recipients were enrolled, and 565 and 398 samples were analyzed in the heart and lung transplant studies, respectively. Post-transplant samples were collected serially at predetermined time points and the proportion of cfDNA was determined. These measurements demonstrated the post-transplant dynamics of cfDNA. For both heart and lung transplants, cfDNA is markedly elevated on postoperative Day 1. For hearts, the fraction of cfDNA drops after Day 1, from up to 10% immediately after the transplant surgery to a very low baseline level ($<0.1\%$) within a few days, and this low baseline is maintained in the absence of rejection. The behavior for lung transplants is notably different: a greater relative proportion of cfDNA is present

in plasma for the first few days after the transplant surgery, with levels up to 50%, and the decay kinetics are much slower—a baseline level on the order of 1% is reached after roughly 60 days post-transplant. cfDNA may be a universal marker of transplant injury that can be used across different transplant settings, but care must be taken to characterize normal dynamics after each type of transplant procedure.

Comparison to endomyocardial and transbronchial biopsy grades provided insight on the diagnostic performance of cfDNA. For both heart and lung transplants, cfDNA was significantly elevated at the time of acute rejection. For hearts, a receiver operating characteristics analysis of the performance of cfDNA in distinguishing moderate-to-severe rejection from quiescence yielded an area under the curve (AUC) of 0.83 (sensitivity of 58%, specificity of 93%, at a threshold of 0.25%). For lungs, a receiver operating characteristics analysis of the performance of cfDNA in

distinguishing moderate-to-severe rejection from quiescence yielded an AUC of 0.9 (sensitivity of 100%, specificity of 73%, at a threshold of 1%). The diagnostic performance in hearts declined with age of the transplant recipients, and improved with time post-transplant. The agreement of cfDNA with biopsy results was particularly strong for pediatric heart transplant recipients, with an AUC of 0.91, consistent with the test performance for pediatric heart transplants reported by Hidestrand and colleagues (10). Lastly, these studies indicated a significant potential for early diagnosis of rejection via cfDNA monitoring: for hearts, the level of cfDNA was significantly elevated up to 5 months before rejection diagnosis by biopsy.

cfDNA is a quantitative marker that can be trended over time (11). This feature may be used to guide therapeutic decisions. For example, the level of cfDNA may be used to inform dose titration of antirejection drugs. Furthermore, cfDNA may be used to assess the response to antirejection therapy. Figure 2 illustrates this point with cfDNA as function of time after initiation of antirejection treatment (six heart transplant recipients diagnosed with severe rejection [data from Reference 9]). In each case, the level of cfDNA decreases in response to therapy, but significant patient-to-patient variability in the response kinetics and in the level of donor DNA at diagnosis are observed. It will be of interest to investigate whether the kinetics of cfDNA decay in response to therapy predicts long-term outcomes or disease relapse.

Significant progress has been made on several fronts recently: alternative measurements of cfDNA based on targeted sequencing (10, 12) and digital polymerase chain reaction (PCR) (13) have been introduced that have the promise of a lower assay cost and faster turnaround time, and recent studies have assessed the performance of cfDNA in a variety of transplant settings, including kidney, pancreas, and liver transplantation (13–15). For an overview of all studies up to 2015, we refer the reader to the review by Gielis and colleagues (16). Although progress has been swift, it will be important to further validate cfDNA in larger cohort studies. The National Institutes of Health has recently created the Genomic Research Alliance for Organ Transplantation (17), with the mission to evaluate cfDNA as a transplant monitoring tool—data from

Figure 2. Donor-specific cell-free DNA (cfDNA) as a measure of therapeutic response. Donor DNA levels after diagnosis and treatment of severe heart transplant rejection (International Society for Heart and Lung Transplantation grade 3R, the most severe rejection grade) (data for six patients, at rejection [red circles] and post-rejection [black circles]). Black line is fit of exponential decay model. Data from Reference 9.

this multicenter initiative will play an important role in further guiding this fast-moving field.

Profiling the Virome via Analyses of cfDNA

Immunosuppressive therapies significantly reduce the risk of rejection, but increase the susceptibility of transplant patients to infection. Diagnosis of opportunistic infections is challenging, given that symptoms of infection are often diminished after immunosuppression, and that many diagnostic tests are sensitive to only a single pathogen. In this context, it is reasonable to ask whether components of the microbiome can be quantified through sequence analysis of cfDNA. A recent study quantified the relative genomic abundance of viral DNA fragments in the plasma of heart and lung transplant recipients (18). By far the most abundant component of the plasma virome consisted of torque teno viruses (TTVs), members of the *Anelloviridae* family. TTV infections are nearly ubiquitous in the human population, with primary infection early in childhood (19). Infection is usually asymptomatic and does not result in any known, clinically significant disease state. For both heart and lung transplants, a striking positive relationship was observed between the abundance of TTV sequences in plasma and overall level of immunosuppression, raising the question of whether TTV loads can be used to gauge immunocompetence. Consistent with this idea, recent studies have shown that TTV loads allow stratification of

patients at risk for rejection (18, 20) and those who are at risk for opportunistic infections (21). Similar relationships between TTV loads in plasma and immunocompetence and immune control of TTV viremia have been reported in the context of AIDS and stem cell transplantation (22–24). Young and coworkers (25) used viral metagenomics of allograft bronchoalveolar lavages, similarly revealing blooms of anelloviruses in the respiratory tract of lung transplant recipients. For a timely review on the use of TTV as a marker of immunocompetence, see Reference 26.

Shotgun sequencing of cfDNA may be useful as a tool to broadly screen for infectious complications after transplantation (27). This concept was investigated in the context of lung transplantation through comparison of cfDNA fingerprints to clinical assays for infectious pathogens across a wide range of sample types (8). This study established an agreement between the load of cytomegalovirus DNA measured by sequencing of cfDNA and clinical PCR assays for cytomegalovirus viral load (AUC = 0.91). Agreements were furthermore found between cfDNA measurements and fungal infections detected in bronchoalveolar lavage specimens, *Klebsiella pneumoniae* detected in urine culture, and microsporidium clinically detected in stool (8). Finally, unbiased sequencing revealed a wide range of undiagnosed infections and, overall, a disconnect between the frequency of clinical testing and the prevalence of

infections, such as polyomavirus and human herpes viruses 4, 6, and 7.

Biophysical Properties and Bias-Free Measurements

cfDNA exists in plasma in many shapes and forms, with fragments derived from all chromosomes, the mitochondrial genome, and microbial genomes (28). Complete degradation of these molecules in plasma is prevented through tight association with DNA-binding proteins. The physical properties of cfDNA depend, to a large extent, on the properties of the cfDNA–protein complex, and differ significantly for microbial, mitochondrial, and chromosomal cfDNA. Chromosomal cfDNA is predominately nucleosomal, with a fragment size centered around 165 bp, approximately the length of a segment of DNA wound around a histone complex. A small fraction of cfDNA is bound to transcription factors, and these molecules are shorter than nucleosomal cfDNA, reflecting the smaller DNA footprints of transcription factors (29). Fragments of mitochondrial and microbial DNA in plasma are significantly more degraded than nucleosomal cfDNA, resulting in fragment lengths that are, on average, shorter than 100 bp (28).

Fragment sizes matter because of the length biases that are inherent to molecular assays. PCR assays, for example, are insensitive to randomly fragmented cfDNA, with fragment sizes close to or smaller than the PCR amplicon length. Conventional sequencing library preparations that are based on double-stranded ligation of sequencing adapters require size-selective steps that eliminate unwanted adapter dimer products. These assays are consequently relatively insensitive to short fragments of cfDNA. We recently applied a single-stranded DNA library preparation method that does not require size-selective steps to the analysis of cfDNA (28). Sequencing after single-stranded DNA library preparation revealed a rich diversity of ultrashort cfDNA types, including microbial cfDNA and mitochondrial cfDNA. Comparison to mitochondrial reference genomes indicated that a significant proportion of mitochondrial cfDNA is donor derived. Donor-specific mitochondrial cfDNA is an interesting potential biomarker of transplant injury given the large number of mitochondria found in each cell, and given

that mitochondrial DNA has been identified as a powerful damage-associated molecular pattern—an endogenous molecule that can activate innate immunity when released during cellular injury (30).

cfDNA Epigenetic Marks Reveal Its Tissues of Origin

Donor DNA is fundamentally a marker of cellular injury in the graft, and does not discriminate between infection- and rejection-related injury, or between different pathways of rejection, such as antibody-mediated rejection or cellular rejection. To obtain a nuanced understanding of the mechanisms that lead to the release of cfDNA in the circulation, it would be helpful to obtain information about the cellular source of this DNA (11). In principle, such information can be obtained through measurements of epigenetic marks of cfDNA, including cytosine methylation marks, hydroxymethylation marks, and the genome-wide occupancy of nucleosomes and DNA-binding proteins. These features are highly cell-, tissue-, and organ-type specific, and, therefore, in principle, enable identification of the cell, tissue, and organ types that contribute cfDNA toward the mixture in

the circulation. Decomposition of the cfDNA mixture into contributing cell types may enable assessment of the relative degree of injury to endothelial and epithelial tissues, or may enable quantification of the relative contributions of different immune cell types—data that, collectively, will provide deeper insight in to the pathogenesis of graft injury. cfDNA tissues-of-origin measurements will furthermore be useful for assessing individual organ injury in multiorgan transplants, and will also have clinical applications outside of solid-organ transplantation, such as for the diagnosis of graft versus host disease in allogeneic bone marrow transplantation.

Several recent studies have focused on epigenetic marks of cfDNA. Sun and coworkers (31) investigated genome-wide cytosine methylation using bisulfite sequencing of plasma DNA, and demonstrated the ability to perform a rough decomposition of organ types that contribute DNA to the mixture in plasma. Snyder and coworkers (29) demonstrated that cfDNA comprises a nucleosomal footprint, and provided early evidence that these footprints can provide information about the tissues of origin

of cfDNA. It is unclear, at this stage, whether these epigenetic measurements of cfDNA can provide sufficient detailed information about the cellular origin of cfDNA to be useful for in-depth analyses of the pathogenesis of transplant rejection, but there is no doubt that this will be an active area for research in years to come.

Conclusions

In conclusion, circulating cfDNA is quickly emerging as a highly versatile tool for the monitoring of the health of solid-organ transplants. High-throughput DNA sequencers are particularly informative read-out tools of cfDNA, with the potential to yield information about acute rejection, infection, and overall immunosuppression from a single assay. We anticipate that more fun and excitement is just around the corner, as more and more concepts from diverse fields, such as data science, genomics, and biophysics, are being applied to cfDNA-based precision transplant monitoring. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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