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Review

Biomarkers in the Management of the Lung Transplant Allograft: A Focus on Donor-derived Cell-free DNA

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Abstract

Advances in the use of molecular biomarkers to guide management of lung transplant recipients are emerging. One such biomarker, donor-derived cell-free DNA (dd-cfDNA), has the potential to alter traditional approaches of lung transplant management, including the detection of acute rejection or infection, surveillance monitoring strategies, risk stratification of vulnerable patient populations and molecular phenotyping of various forms of allograft injury. The emergence of novel genetic and epigenetic approaches utilizing cfDNA provides further promise for the elucidation of pathophysiological mechanisms and treatment targets in various disease states. This review aims to employ the current state of the evidence to cover the biological basis, clinical applications and future directions for the use of dd-cfDNA in lung transplant recipients.

Keywords
Lung transplant; molecular biomarkers; acute lung allograft rejection

1. Introduction

Lung transplantation remains a viable option for select patients with progressive, end-stage lung disease. Despite this, the median survival after lung transplant remains only 6.5 years, the lowest among solid organ transplant [1]. Episodes of allograft injury, often in the form of acute rejection (AR) and infection, are not only deadly, but may predispose the patient to chronic lung allograft dysfunction (CLAD), the primary cause of long-term mortality post-transplant [2-5]. AR and infection are common after lung transplant, with an incidence of up to 50% and 33%, respectively, in the first post-transplant year [6-8]. Consequently, methods of adequately detecting underlying allograft injury and routinely surveilling lung allograft health are critically important to improving long term outcomes in lung transplantation.

Standardized methods of detecting underlying allograft injury include routine pulmonary function testing (PFT), frequent clinic visits, testing for donor specific antibodies (DSA), and most conspicuously, the use of bronchoscopy with bronchoalveolar lavage (BAL) and transbronchial biopsy (TBBx). However, these tools have limitations. Acute rejection or infection may manifest in the absence of changes in pulmonary function testing or the development of signs and symptoms of allograft dysfunction [9]. Histopathological analysis of TBBx samples to diagnose acute rejection demonstrates high interobserver variability or may be missed altogether if samples are taken from unaffected lung [10]. Further, bronchoscopy with TBBx is a costly procedure, may be inconvenient to the patient and place the patient at risk for procedural complications. Given these challenges, recent investigation has focused on the development of highly sensitive and less invasive methods of detecting allograft injury.

Plasma donor-derived cell free DNA (dd-cfDNA) is a novel, molecular marker of allograft injury that has been the subject of much research in recent years as a potentially accurate, non-invasive method of detecting allograft injury. This review serves to provide insight into the biological basis for its use, measurement techniques and clinical applications to diagnosis, surveillance and risk stratification in lung transplant patients.

2. Identification and Quantification of dd-cfDNA

Cells undergoing apoptosis and necrosis release fragments of double-stranded DNA into bodily fluids, including the bloodstream, known as cell free DNA (cfDNA). The half-life of cfDNA is approximately between 16 minutes and 2.5 hours and it is predominantly excreted in the urine or undergoes local degradation by macrophages in the liver or spleen [11-13]. Levels of cfDNA are generally low in normal physiologic states but increase in the setting of tissue injury. Solid organ transplant creates a unique scenario in which there is also genetic admixture between the donor and recipient. In this setting, it is possible to genetically differentiate cfDNA originating from the donor allograft versus cfDNA originating from recipient cells. Therefore, measuring donor derived

cell free DNA (dd-cfDNA) may provide specific insight into the degree of allograft tissue injury, including in the setting of acute rejection or infection.

Laboratory techniques developed to measure levels of plasma dd-cfDNA rely on two principle components: 1) methods to identify donor vs. recipient genetic material and 2) methods to quantify or semi-quantify the amount of dd-cfDNA. Early assays relied on sex-based chromosomal differences or degree of HLA mismatch between the donor and recipient, however, these were limited by the requirement for gender or HLA mismatch between the donor and recipient, respectively, to distinguish the genetic material [14, 15]. More contemporary assays utilize differences in single nucleotide polymorphisms (SNPs) between donor and recipient to identify their respective cell free DNA. Multiple SNP-based techniques have been developed. First generation two-genome approaches genotyped donor and recipient using DNA to identify informative SNPs [16]. The subsequent "one-genome" approach genotyped recipient DNA, and used this to infer informative SNPs [17]. The "one genome" approach could thus be utilized when donor DNA is lacking. Nonetheless, the need for genotyping is time consuming, potentially limiting its use of these approaches in routine clinical practice. More recent, and now commercially available, techniques involve leveraging publicly available population genomic data to define informative SNPs that are representative in different patient populations, obviating the need for genotyping of recipient and pre-transplant donor DNA [18, 19].

Once donor vs. recipient SNPs have been identified, several methods exist to amplify and quantitate the amount of cfDNA present to provide a measure of plasma dd-cfDNA. These techniques include whole genome sequencing, quantitative polymerase chain reaction (PCR), digital droplet PCR or targeted sequencing [20, 21]. While absolute amounts of dd-cfDNA may be reported, differences in the size of the allograft as well as single vs. double lung transplants may interfere with the interpretation of these results, because differences in baseline absolute levels of dd-cfDNA would depend on the total amount of allograft tissue. Therefore, dd-cfDNA is traditionally presented as a percentage of dd-cfDNA over total cfDNA (dd-cfDNA + recipient cfDNA). Notably, because the amount of recipient cfDNA lies in the denominator of this calculation, fluctuations in recipient cfDNA may alter levels of %dd-cfDNA, potentially affecting interpretations of the results. Prior research also indicates that correcting %dd-cfDNA in single lung transplant recipients by doubling the value in order to account for the reduced allograft tissue mass, eliminated the differences in dd-cfDNA between double vs. single lung transplant patients and preserved performance characteristics of the test to detect AR at common threshold values [22]. Research is ongoing to determine the optimal method of reporting levels of dd-cfDNA while best accounting for both allograft size and amount of recipient cfDNA.

3. Clinical Applications

As a molecular biomarker of underlying allograft injury, dd-cfDNA has the potential for broad clinical applications in the management of lung transplant recipients. While the majority of evidence has investigated the utility of dd-cfDNA to detect episodes of acute rejection and infection, additional evidence supports the potential role for dd-cfDNA in other domains, including risk stratification and tailoring treatment of immunosuppressive drugs.

3.1 Detection of Acute Rejection and Infection

Levels of dd-cfDNA are significantly elevated immediately following the transplant surgery, after which, they decay logarithmically in a two-phase decay pattern to reach stable baseline levels around 3 to 6 months post-transplant [23, 24]. While stable patients may follow this characteristic pattern of decay and baseline, multiple studies have demonstrated that dd-cfDNA increases in the setting of acute rejection and infection [25-28]. In 2015, De Vlaminck et al. performed a single center, prospective cohort study in which they longitudinally collected serial plasma samples for dd-cfDNA at fixed time points in 51 lung transplant recipients. Samples were also obtained at the time of bronchoscopy [23]. In comparison to stable controls, dd-cfDNA significantly increased at the time of moderate or severe ACR (histopathological ACR grade ≥3). A dd-cfDNA threshold level of >1% demonstrated an area under the receiver operating curve (AUC) of 0.9 for the detection of moderate or severe ACR, with a sensitivity of 100% and specificity of 73%.

In 2021, Jang et al. performed a multicenter, prospective cohort study in which they monitored 148 lung transplant recipients over a median of 19.6 months [27]. Serial plasma samples were collected to measure dd-cfDNA at the time of both clinically indicated and surveillance bronchoscopy with BAL/TBBx. ACR was defined as histopathological grade ≥A2 or ≥A1 with corresponding decline in the forced expiratory volume in 1 second (FEV1) ≥10%. Median levels of dd-cfDNA were significantly elevated in patients with acute rejection (ACR and clinical AMR) vs. stable controls (1.95% vs. 0.30%, $p < 0.01$). A dd-cfDNA threshold of ≥1% yielded an AUC of 0.89 for the detection of AR, with a sensitivity of 77%, specificity or 84%, positive predictive value (PPV) or 60% and negative predictive value (NPV) of 90%. Notably, levels of dd-cfDNA appeared to rise prior to the diagnosis of AMR, with 82% of AMR episodes demonstrating a rise ≥1% one month prior to the diagnosis. Further, this study also demonstrated that levels of dd-cfDNA were elevated in the presence of infection, defined as positive microbiology plus either an acute decrease in FEV1 ≥10% or histopathological changes consistent with allograft injury, in comparison to stable controls.

Given the encouraging performance characteristics of dd-cfDNA for the detection of acute rejection, including the high negative predictive value reported in observational studies and potential rise in levels prior to clinical manifestations of rejection, it has potential utility in post-transplant surveillance protocols, perhaps in place of surveillance bronchoscopy with BAL/TBBx. A recent multicenter, retrospective cohort study demonstrated the relative safety and feasibility of this approach in the real-world setting [28]. In this study, in an effort to mitigate the risk of COVID-19 infection at the onset of the COVID-19 pandemic, four lung transplant centers deferred performance of surveillance bronchoscopy, routine clinic visits and clinic-based PFTs, and instead, enrolled patients in a home-based monitoring program that utilized serial testing of dd-cfDNA to monitor for acute rejection or infection. Over the first 6 months, the program enrolled 198 patients who received over 400 plasma samples for dd-cfDNA. Using a threshold value of ≥1% to perform bronchoscopy, twenty-three episodes of asymptomatic acute rejection or infection necessitating treatment were identified. Ninety-six percent of patients with a dd-cfDNA level <1% remained stable over the course of study, defined as the absence of acute rejection, infection, development of signs or symptoms of allograft dysfunction or PFT decline over the course of the study period with at least 1 month follow up to ensure stability. Further, the number of bronchoscopies performed was reduced by 82% compared to the expected amount.

In summary, levels of dd-cfDNA increase in the setting of acute rejection and infection and demonstrate good performance characteristics for the detection of underlying allograft injury. Additional research is required to determine optimal dd-cfDNA threshold levels for detection of specific allograft injury states. Further, it is important to highlight that dd-cfDNA is released in reponse to various forms of allograft injury and is not necessarily specific to the precise *cause* of allograft injury (i.e. rejection vs infection). Recent studies have demonstrated the potential for incorporating cfDNA fragment size profile with specific dd-cfDNA threshold levels to differentiate between various allograft injury states [29]. The available evidence also supports further investigation into the role of dd-cfDNA in surveillance monitoring, including a randomized controlled trial comparing a dd-cfDNA-based surveillance method to traditional surveillance bronchoscopy [30].

3.2 Detection and Prediction of Chronic Lung Allograft Dysfunction

While several studies have investigated the ability of dd-cfDNA to detect episodes of acute rejection and infection, studies supporting its utility in the detection of CLAD are sparser. In a single center prospective cohort study, Khush et al. reported higher levels of dd-cfDNA in patients with the obstructive phenotype of CLAD in comparison to normal controls [31]. In another single center retrospective cohort study, Yang et al. demonstrated higher levels of total cfDNA in the BAL fluid in patients with CLAD (n = 40) vs. stable controls (n = 20) [32]. In addition, they demonstrated that higher levels of cfDNA in the BAL fluid in patients with CLAD increased the risk of mortality. However, while these studies support the association of CLAD with increases in cfDNA, CLAD remains a disease without viable treatment options, and therefore, the ability to predict CLAD before its onset may be more clinically useful. The ability to adequately predict and risk stratify patients for the development of CLAD may allow clinicians to tailor surveillance monitoring strategies, more aggressively treat/address other risk factors for CLAD and identify at-risk patients that may benefit from inclusion in clinical trials aimed at the prevention of CLAD.

In 2019, our lab performed a multicenter, prospective cohort study demonstrating that elevations in dd-cfDNA in the early post-transplant period increased the risk of CLAD and death [24]. In this study of 108 lung transplant recipients with a median follow-up of 36 months, three distinct patterns of dd-cfDNA decay kinetics were observed that varied among individual subjects during the first 90 days post-transplant. The low tercile group demonstrated a rapid decline in dd-cfDNA to low, stable levels within one-month post-transplant. The middle tercile displayed a slower decay but reached baseline levels similar to the low tercile group by 90 days post-transplant. The upper tercile group had the slowest decay, with persistently elevated dd-cfDNA levels compared to the other two groups. Subjects in the upper tercile group had a 7.8-fold higher risk of allograft failure.

Other studies have demonstrated similar associations between prior molecular evidence of allograft injury and the downstream risk of CLAD. In a multicenter, prospective cohort study, levels of dd-cfDNA at the time of diagnosis of primary graft dysfunction (PGD) increased the risk of CLAD, irrespective of clinical PGD severity grade [33]. Similarly, Bazemore et al. demonstrated that levels of dd-cfDNA at the time of diagnosis of respiratory viral infections increased the risk of CLAD and death [34]. Early identification and risk stratification of these vulnerable patient populations may be key to developing preventative strategies for CLAD and improving long term outcomes.

4. Future Directions

Several advances are being made in the field of cfDNA and solid organ transplantation. Among the most important are identifying the cfDNA tissue of origin using tissue-specific epigenetic signatures and exploring the role of cfDNA as a potentiator of tissue injury and potential therapeutic target.

4.1 Cell-free DNA Tissue of Origin

While dd-cfDNA is released into the blood stream in response to cellular necrosis or apoptosis, it is not necessarily specific to the *cause* of tissue injury. As stated earlier, various injury states in the allograft can cause increases in dd-cfDNA including ACR, AMR, CLAD and infection. Further identification involves incorporating bronchoscopy with BAL/TBBx, microbiology, donor specific antibody testing and PFTs to diagnose the specific cause of allograft injury. However, these tools may also be non-specific. For example, biopsy has been demonstrated to show overlapping histopathological features for ACR and AMR [35]. The ability to further differentiate the precise tissue injury pattern in these diseases would greatly enhance the ability to non-invasively diagnose allograft dysfunction and provide clearer mechanistic insight into the pathophysiology of various disease states.

Although all cells in the recipient may share the same genome sequence, their epigenetic fingerprints are distinct, including tissue-specific DNA methylation patterns, histone modifications and chromatin footprints. These signatures are surveyed on cfDNA to assign the tissue of origin [36-38] (Figure 1). cfDNA fragment production is potentially non-random, but guided by the tissue source and the underlying biological processes. Recent studies indicate that the fragment characteristics vary with disease states and potentially tissue of origin. This suggests that fragment size, jagged end or end motifs can be utilized to infer the biological processes associated with various disease states [39-41]. For example, the GRAfT Investigators showed that cfDNA fragment length and GC content differ between AMR and ACR in heart transplants recipients [42].

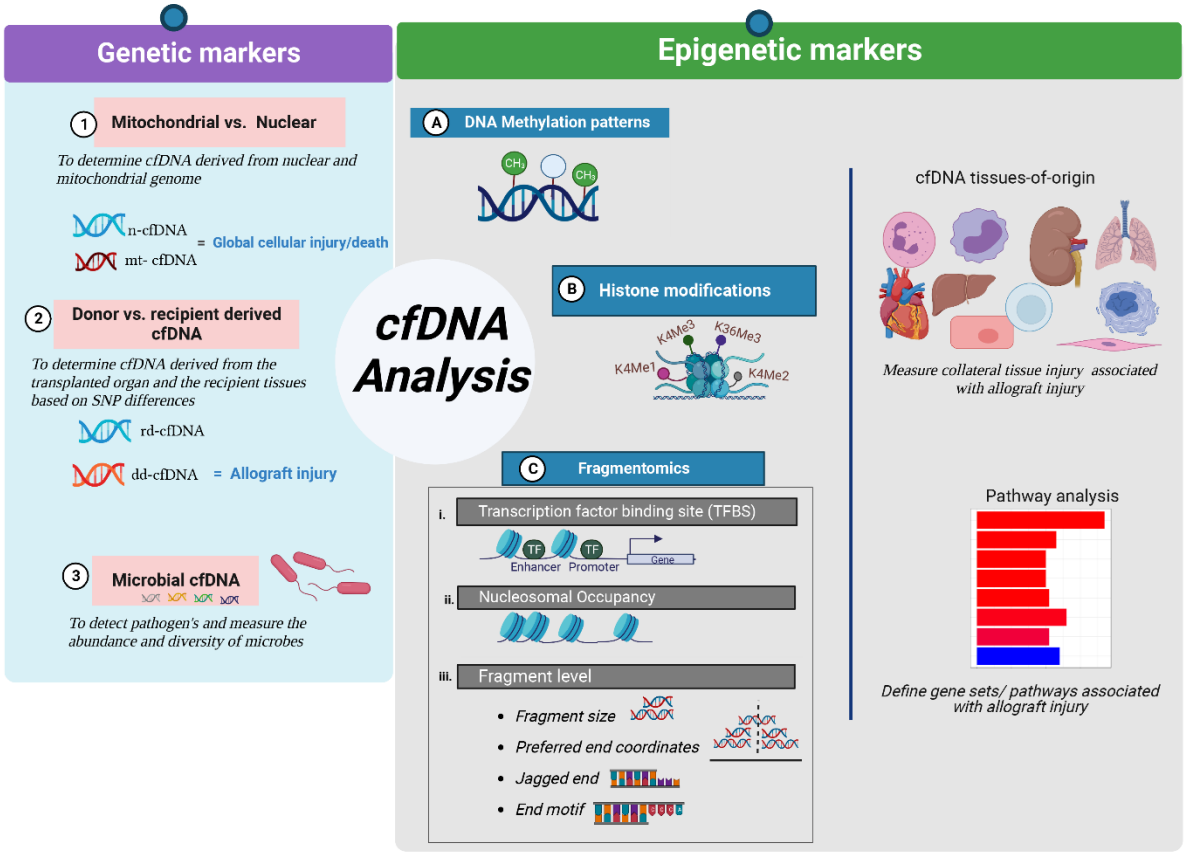

Figure 1 Overview of the potential genetic and epigenetic approaches associated with the analysis of cell free DNA. Differences in donor vs. recipient single nucleotide polymorphisms may be used to genetically differentiate dd-cfDNA from recipient cfDNA and quantitate the degree of allograft injury. Mitochondrial cfDNA may be isolated and quantified, and have important implications on the pathogensis of various disease states, particularly as a damage associated molecular pattern. Detection of microbial cfDNA may provide novel approaches to the diagnosis of infection as well as further insight into the diverse landscape of the microbiome. A host of epigenetic techniques allow for the precise identification of cfDNA tissue of origin, expanding our knowledge of the pathophysiological mechanisms of various transplant-associated disease states.

Using bisulfate sequencing techniques to identify tissue specific DNA methylation signatures, Andargie et al. effectively provided whole body mapping of tissue-injury patterns in a cohort of COVID-19 patients, providing further mechanistic and prognostic insight into course of the disease [43]. Brusca et al. applied a similar approach to define biologically plausible tissue injury sources that predict transplant-free survival in patients with pulmonary arterial hypertention [44]. Similarly, the ability to apply such tissue mapping techniques to lung transplant patients may allow for better identification of acute and chronic rejection phenotypes, mechanisms of disease and therapeutic targets.

4.2 Microbial cfDNA

Microbial infection is another major complication after lung transplantation. Metagenomic studies demonstrate that DNA fragments from microorganisms, called microbial cfDNA (mcfDNA),

can be detected in the peripheral blood and used as a potential biomarker to monitor infection [45, 46]. Recently, Blauwkamp et al. developed a microbial cfDNA (mcfDNA) sequencing assay called Karius Test™ that detects over 1000 clinically relevant pathogens, including bacteria, DNA viruses, fungi, and eukaryotic parasites, rapidly and non-invasively [47]. This test showed high agreement with gold-standard blood culture methods in patients with sepsis [47]. This approach may also help detect clinically relevant pathogens in lung transplant patients. In addition, metagenomic sequencing of mcfDNA, that measures the abundance and diversity of human virome, may be used as a surrogate marker of immunosuppression in transplant patients [48].

4.3 Cell-free DNA as a Biological Trigger of Tissue Injury

Despite the abundance of evidence profiling elevations in cfDNA in response to tissue injury, accumulating evidence also implicates cfDNA as a potential *trigger* of tissue injury. cfDNA has been implicated as a damage-associated molecular pattern (DAMP) that is recognized by pattern recognition receptors (PRRs), which in turn, activate immune signaling and cell death pathways that provoke tissue injury. Through Toll-like receptor 9 (TLR9) mediated pathways, circulating cfDNA and extracellular mitochondrial cfDNA have been shown to provoke acute lung injury and acute kidney injury in mouse models [49-51]. Inhibition of TLR9 attenuated the inflammatory response in these models [52, 53]. Similarly, in mice lung transplantation models, the release of mitochondrial cfDNA into the transplanted lung as a result of ischemia-reperfusion injury triggered TLR-9 dependent enhanced lung injury mediated by neutrophil extracellular traps (NETs) [54], which contain cfDNA. Accordingly, DNase I therapy has been demonstrated to attenuate lung injury in murine PGD models by disrupting NETs [55]. This data suggests that cfDNA released during various forms of allograft injury may further potentiate allograft damage and increase disease severity. Further, cfDNA may serve as a therapeutic target to alleviate the extent of allograft injury in various disease states.

5. Conclusion

As a non-invasive marker of underlying allograft injury at the molecular level, dd-cfDNA provides a wealth of potential clinical and investigational applications in the field of lung transplantation. While still in the early stages of investigation into realizing its clinical utility, advances in the ability of dd-cfDNA to detect episodes of allograft dysfunction, identify and risk stratify patients at risk for adverse outcomes, and provide mechanistic insight into the pathophysiology of various disease states furthers the goal of ushering lung transplantation into the world of precision medicine.

Author Contributions

Dr. Michael Keller, Dr. Sean Agbor-Enoh and Dr. Temesgen Andargie all contributed in the writing and review of this manuscript. Dr. Temesgen Andargie developed all of the figures for this manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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