A Noninvasive and Donor-independent Method Simultaneously Monitors Rejection and Infection in Patients With Organ Transplant

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ABSTRACT

Background. Rejection and infection are 2 major complications affecting the health and survival of patients receiving an allograft organ transplantation. We describe a diagnostic assay that simultaneously monitors for rejection and infection in recipients of kidney transplant by sequencing of cell-free DNA (cfDNA) in plasma.

Methods. By using cfDNA in plasma, we established a noninvasive method that simultaneously monitors rejection and infection in patients with a history of organ transplant. A total of 6200 single-nucleotide polymorphisms were captured by liquid hybridization and sequenced by next-generation sequencing. The donor-derived cfDNA (ddcfDNA) level was calculated based on maximum likelihood estimation, without relying on the donor's genotype. We also analyzed the nonhuman cfDNA to test for infections in the patients' plasma.

Results. Artificial ddcfDNA levels quantified by a donor-dependent and donor-independent algorithm were significantly correlated, with the multivariate coefficient of determination, or R^2 value, of 0.999. This technique was applied on 30 patients (32 samples) after kidney transplantation, and a significant difference was observed on the ddcfdNA levels between nonrejection and rejection. Furthermore, 1 BK virus infection and 1 cytomegalovirus infection were revealed by this method, and the enrichment efficiency of the viral sequences was 114 and 489 times, respectively, which are consistent with clinical results.

Conclusion. This method can be used to simultaneously monitor for acute rejection as well as a broad spectrum of infections for patients of allograft organ transplant because it provides comprehensive information for clinicians to optimize immunosuppression therapy.

Organ transplantation is considered as an ultimate option for the treatment of various end-stage organ diseases, and as a result, a lifetime surveillance of grafted organ health and monitoring for infection are essential for the survival of the patient [1–3]. Immunosuppression drug, such as tacrolimus, which reduces the occurrence of graft rejection by suppressing the immune system, can also increase the risk of infection in patients with a history of allograft transplantation [4]. Biopsy is regarded as the "gold standard" in the diagnosis of graft rejection and infections. However, it is not a suitable method for the constant

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monitoring of transplanted organ health since it requires regular organ excisions; due to its invasive nature, it can cause a certain level of damage to the graft organ [5]. Diagnosis of infection requires the treating physician to correctly identify the infection source and to order a series of pathogen-specific tests. Posttransplantation infection and rejection often manifest similar symptoms, such as shortness of breath, and standard clinical tests often fail to distinguish between these 2 common complications [2].

Cell-free DNA (cfDNA), that is, fragmented nucleotides circulating in the plasma, was first report in 1948, and since has been widely applied in the field of noninvasive prenatal test and liquid biopsy for cancer [6–9]. The donor-derived cfDNA (ddcfDNA) is found in the recipient's plasma in various types of solid organ transplantations, and is considered as a noninvasive and real-time biomarker for monitoring the occurrence of acute rejection of transplanted organs [10–12]. To date, a number of technologies have been used to quantify ddcfDNA levels in recipients of organ transplant, such as quantitative polymerase chain reaction (PCR), next-generation sequencing (NGS), and donor-derived PCR [13–16]. Previous studies used Y-chromosome specific alleles to quantify the ddcfDNA, but this technique could only be performed on a quarter of the patients with history of organ transplantation [17,18]. Recent methodologies applying donor-derived PCR or NGS to quantify the ratio of donor-specific single-nucleotide polymorphism (SNP) in recipients' cfDNA provide universal methods for patients of allograft organ transplant, but techniques for simultaneously monitoring both acute rejection and infection in recipients are scarce. De Vlaminck et al [2] employed cfDNA whole-genome sequencing to monitor both acute rejection and infection in recipients after lung transplantation, but this method depends on donor genotype, which is not available for many patients with allograft organ transplants.

cfDNA circulating in the blood plasma is extremely low in concentration, with only a few thousand genomic copies per milliliter; therefore, its use as a diagnostic marker requires precise methods of quantification. In this study, we developed liquid hybridization technology and NGS to simultaneously monitor acute rejection and infection in patients with a history of allograft transplantation. The donor genotype is not necessary for this technique to accurately quantify the ddcfDNA level in recipient's plasma. Targeted 20 organ transplant–related pathogenic microorganisms, including viruses, bacteria, fungi, and protozoa can be detected by sequencing the species-specific genome region. This assay is noninvasive and comprehensive, and can therefore serve as an ideal constant health surveillance tool for patients of allograft organ transplants.

MATERIALS AND METHODS

Blood Collection and Cell-free DNA Library Construction

The study was reviewed and approved by the ethics committee of Second Xiangya Hospital of Central South University, with a reference number of ethical approval (2017–155). All the peripheral blood samples were performed after obtaining fully informed consent from all patients with kidney transplants.

A peripheral blood sample (8 mL) was collected from patients with kidney transplants by using the cfDNA blood collection tubes cfDNA BCT (Streck, Ne, United States). For patients who performed tissue biopsy, the blood sample was drawn on the same day. Whole blood was centrifuged at 1600*g* at 4°C for 10 minutes. Plasma was transferred into sterile Eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuged again at 16,000*g* at 4°C for 10 minutes, and the supernatant was preserved at -80°C until the time of using. From 1.8 mL plasma, cfDNA was isolated using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, Calif, United States) by following the manufacturer's protocol. The purified cfDNA was then quantified by Qubit fluorometer (Life Technologies, Carlsbad, Calif, United States). A cfDNA library was constructed by applying KAPA LTP Library Preparation Kit (Kapa Biosystems, Wilmington, Mass, United States), with 10 to 50 ng of cfDNA input for each sample and 8 PCR cycles for library amplification.

Standard Curve Construction

To validate the accuracy of our ddcfDNA quantification method, we diluted a human genomics DNA sample (artificial donor, A1) with another biologically unrelated specimen (artificial recipient, A2) into a series of concentrations: 0.6%, 2%, 4%, 6%, 8%, and 10% in triplicate. An NGS library was constructed on those 18 mixed genomic DNA samples by using the transposase-based library construction kit TruePrepTM (Vazyme Biotech, Nanjing, China).

Target Region Enrichment and Sequencing

A total of 6200 human SNP loci as well as 13-Kb species-specific regions of 20 pathogens were enriched by liquid hybridization. The 6200 SNPs were selected based on the following standards: 1. high polymorphism in Chinese population; 2. single copy in the genome; and 3. even distribution across the whole genome. The targeted 20 pathogens were those reported to be related to high incidence of infection in patients of organ transplants, such as BK virus, cytomegalovirus (CMV), and *Klebsiella pneumoniae*. Eighty nucleotide oligomer probes, with GC content between 40% and 50%, were designed on those targeted area by a self-developed pipeline. Flank sequences (GGGATGAGACAGGGGAGATA-insert- GCCATACACAGGACTGTCAT) were added to each probe, making the DNA probe 120 nucleotides. PCR-amplified DNA probes were used as templates to generate biotin-labeled RNA baits by using in vivo transcription kit (Invitrogen, Carlsbad, Calif, United States). An aliquot of 500-ng cfDNA library was incubated with 500-ng RNA probes in hybridization buffer (10X sodium chloride-sodium phosphate EDTA, 10X Denhardt solution, 10 mM EDTA, and 0.2% sodium dodecyl sulfate) at 65°C for 18 hours. M-280 streptavidin Dynabeads (Invitrogen) was added to the hybridization mix to extracted targeted sequences, which were subsequently amplified and purified for sequencing. Each enriched library was sequenced for 5 million paired-end 150 clusters by HiSeq X Ten Sequencing System (Illumina, San Diego, Calif, United States).

Bioinformatics and ddcfDNA Quantification

Sequencing raw data were trimmed by removing adaptors, low-quality reads, and PCR duplications. Clean data were subsequently aligned to the Genome Reference Consortium Human

Build 38 (human reference genome GRCh38) by using the Burrows-Wheeler Aligner program (SourceForge.net, Dice Holdings, New York, New York, USA). SNPs with the sequencing depth below 30X were discarded. For each of the 6200 SNPs, the reads number were counted for each allele, and minor allele ratio value, a_i, for the SNP locus was then calculated:

a_i = minor allele counts/total allele counts for the SNP.

The minor allele ratio value a_i is always below 0.5. According to the distribution of a_i, the value of the peak next to 0 is designated as initial estimated value of ddcfDNA concentration, C_0. SNPs with a_i below $2C_0$ are called as homozygous for recipients, if C_0 is below 10%. Additional SNP genotyping must be carried out on recipients' white cell DNA if C_0 is above 10%. Recipients' homozygous SNP loci with at least one alternative allele read are considered as informative SNPs, which are used for the following donor-independent ddcfDNA quantification. A maximum likelihood estimation–based approach was applied to quantify the ddcfDNA level - λ.

$$L(\lambda) = \Pi p(x_i;\lambda)$$

We adopted a binomial model to estimate the value of λ for each SNP locus. When the donor genotype is available, $x_i = a_i$ if the donor is homozygous for this theoretical informative SNP, and $x_i = 2a_i$ if the donor is heterozygous. As for the donor-independent method, the value of x_i for one particular informative SNP_i is:

$$x_i = paa \times a_i + pAa \times 2a_i$$

paa is the homozygous frequency of the sequenced minor allele in Chinese population, and *pAa* is for the heterozygous frequency. $L(\lambda)$ is then transferred to $l(\lambda)$:

$$l(\lambda) = \Sigma \ln p(x_i;\lambda)$$

The extreme maximum value of $l(\lambda)$ is calculated by the general optimizer nlminb() function in R (The R Foundation for Statistical Computing, Vienna, Austria).

Pathogen Identification

We applied Kraken (Johns Hopkins University, Baltimore, Md, United States) as the taxonomy classifier by mapping k-mers of query sequence to the lowest common ancestor of all reference genomes. According to the algorithm of Kraken, sensitivity and accuracy highly depends on database size, so we built a custom database that includes 984 bacteria species, 248 fungus species, 657 viruses, 34 parasites, and the human genomes GRCh38. SAMtools (http://samtools.sourceforge.net/) was employed to calculate the depth of each pathogens genome sequence. Regions that overlapped with probe sequence were treated as the on-target area, and those outside the on-target area (as well as the flanking 500 bp regions) were considered the off-target area.

RESULTS

The Enrolled Patients

From June of 2017 until July of 2018, 30 patients with history of renal transplant were enrolled. Thirty-two samples from 30 patients were analyzed. All rejections and episodes were based on clinical evaluation and confirmed by renal biopsy.

Sequencing Data of Artificially Mixed DNA Samples.

Targeted sequencing was performed on 18 artificially mixed genomic DNA samples. On average, 5.2 million clusters were generated for each specimen, and the mean sequencing depth of the targeted 6200 SNPs was 119.3X. Sequencing error (0.043% ± 0.0097%) was estimated by calculating the alternate allele in AA + AA combination SNPs according to artificial donor (A1) and recipient (A2) genotype. The theoretical informative SNPs comprised of 1297 SNPs with AA in A1, and Aa or aa in A2. Donor-independent method employed emerged informative SNPs for ddcfDNA quantification. The percentage of emerged informative SNPs was significantly related to mixed ddcfDNA level under the existing sequencing depth (Fig 1).

Donor-independent Algorithm

The level of A1 DNA in each sample was quantified by both donor-dependent and donor-independent methods, and the 2 measured concentrations were plotted against each other in Table 1. The R^2 value of the fitness line is 0.998.

According to the 6200 SNPs genotypes in A1, we categorized informative SNPs into homozygous (Hm, N = 328) and heterozygous (Ht, N = 969) group, which were used to calculate the A1 level separately. The R^2 value of the fitness line is 0.999, with a slope of 1.01 (Table 2). It indicates that this algorithm is robust when the combination of heterozygous and homozygous informative SNPs varies, even if the donor and recipient are biologically related and with 100% heterozygous informative SNPs.

ddcfDNA Levels in Patients With Renal Transplants

To assess the validity of ddcfDNA as a biomarker of rejection in recipients of organ transplants, plasma levels of ddcfDNA were determined in 30 recipients of renal transplant (32 samples) with a biopsy-confirmed rejection and compared to levels of ddcfDNA in recipients of renal transplant without rejection. The mean ddcfDNA ratio of the 32 samples was 1.17%, and

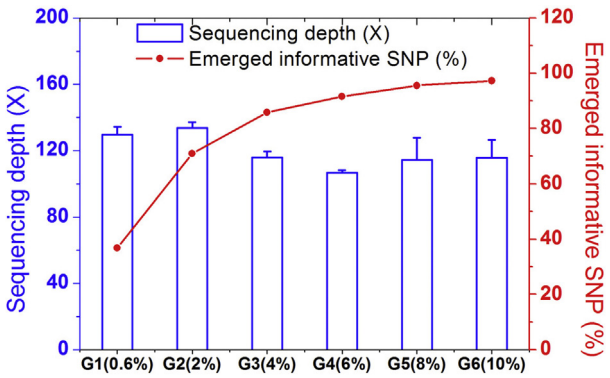

Fig 1. Sequencing depth and percentage of emerged informative single-nucleotide polymorphisms (SNPs) of each A1/A2 mixed group.

Table 1. Artificial Donor-Derived Cell-free DNA Levels Quantified by Donor-dependent and Donor-independent Algorithm

Sample	Theoretical ddcfDNA level (%)	Artificial ddcfDNA Level (%)		R^2 Value (DD, DI)
		DD (N = 3)	DI (N = 3)	
G1	0.6	0.60 ± 0.0042	1.12 ± 0.021	0.998
G2	2	1.74 ± 0.010	2.20 ± 0.14	
G3	4	3.63 ± 0.048	4.17 ± 0.089	
G4	6	5.46 ± 0.031	6.27 ± 0.088	
G5	8	7.33 ± 0.10	8.16 ± 0.034	
G6	10	9.39 ± 0.25	10.19 ± 0.20	

Values are given in mean ± SD unless otherwise specified.
Abbreviations: DD, ddcfDNA level quantified by donor-dependent method; ddcfDNA, donor-derived cell-free DNA; DI, ddcfDNA level quantified by donor-independent method.

the highest and lowest ddcfDNA level was 3.53% and 0.23%, respectively. Eleven out of the 32 patients were classified as rejection by biopsy. The ddcfDNA levels in patients with rejection (1.69% ± 0.79%) were revealed to be significantly higher than those in the comparator group (0.90% ± 0.38%) of biopsy specimens without rejection ($P = .0036$) (Fig 2).

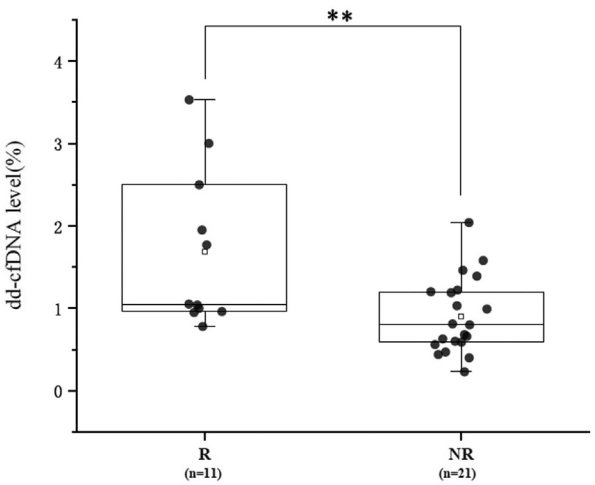

Fig 2. Donor-derived cell-free DNA (ddcfDNA) levels in patients experiencing rejection as determined by biopsy pathology (rejection [R], n = 11) compared to samples from patients with no evidence of rejection (no rejection [NR], n = 21).

Infection Detection in Patients With History of Transplant

The targeted sequencing data from 30 patients with allograft transplants (32 samples) were also analyzed for pathogen infection. Two occasions (QY29, QY30) of virus infection, 1 by BK virus and the other by CMV, were revealed by this study. Both samples were clinically validated by quantitative PCR. The data from targeted sequencing method were categorized into on-target and off-target reads, which are displayed separately in Table 3. In addition to the targeted sequencing methods, whole-genome sequencing (WGS) was also carried out on the 2 specimens. All 3 detection methods successfully identified corresponding pathogens in both samples. On-target data had the highest percentage of viral sequences in both samples among the 3 methods. For the BK virus–infected sample, the mean sequencing depth of on-target and off-target area was 4982X and 43.63X, respectively, which implies that the enrichment efficiency of the BK virus–specific probes is 114 times. As for CMV probes, the enrichment efficiency is 489 times (Table 3).

Table 2. Artificial Donor-Derived Cell-free DNA Levels Measured by Using Heterozygous and Homozygous Informative Single-Nucleotide Polymorphisms

Sample	Theoretical ddcfDNA level (%)	Artificial ddcfDNA Level (%)		R^2 Value (Hm, Ht)
		Hm (N = 3)	Ht (N = 3)	
G1	0.6	0.58 ± 0.090	0.61 ± 0.055	0.999
G2	2	1.63 ± 0.055	1.75 ± 0.062	
G3	4	3.30 ± 0.076	3.55 ± 0.053	
G4	6	5.19 ± 0.12	5.34 ± 0.043	
G5	8	7.02 ± 0.10	7.15 ± 0.24	
G6	10	8.88 ± 0.45	8.86 ± 0.19	

Values are given in mean ± SD unless otherwise specified.
Abbreviations: ddcfDNA, donor-derived cell-free DNA; Hm, donor, homozygous; Ht, donor, heterozygous.

Viral sequences from WGS and targeted sequencing were aligned to the corresponding pathogen genome (Fig 3). Targeted sequencing data were mapped to the whole genome and masked genome, respectively. For the BK virus–infected sample, off-target reads covered 96.23% of the masked BK virus genome, and WGS reads covered 99.73% whole BK virus genome. As for data from patients infected with CMV, off-target and WGS reads covered 13.01% and 28.96% of their corresponding genome regions, respectively. A certain level of similarity was observed in the depth distribution of masked genome between WGS and the targeted sequencing reads from the BK virus–infected sample, and the correlation was found to be statistically significant ($P < .001$, Pearson analysis).

DISCUSSION

ddcfDNA has been considered as a promising biomarker for indicating the occurrence of acute rejection in various types of organ allograft since it was first reported in 1998 [19,20]. The halftime of cfDNA in vivo is approximately 16 minutes [21], however, cfDNA is quite stable in blood after been drawn out of the body, which makes cfDNA a real-time biomarker and it is also easy to be preserved and transported [22,23]. Methodologies using SNP as genetic biomarkers to quantify ddcfDNA levels have been increasingly developed in recently years, mainly due to the advent of new molecular technologies such as digital PCR and NGS [14,24,25].

There are 2 major obstacles for developing a donor-independent ddcfDNA quantification method: 1. differentiating donor and recipient genotype and 2. quantifying ddcfDNA using mixed but unidentified donor-homozygous and donor-heterozygous informative SNPs. Targeted

Table 3. Data Statistics of Sequenced Viral Fragments in 2 Infected Samples

Case	Virus Identified	Methods	Genome Region (bp)	Clean Reads (M)	Viral Reads	Percentage (%)	Coverage (%)	Mean Depth (X)
1	BK	On-target	289	0.76	34,852	4.59	100	4982.0
		Off-target	3134	27.4	22,661	0.083	96.23	43.63
		WGS	5153	80.0	11,559	0.145	99.73	222.72
2	CMV	On-target	1293	0.73	3805	0.52	100	137.01
		Off-target	228007	29.5	393	1.33e-03	13.01	0.28
		WGS	235640	52.0	1251	2.40e-03	28.96	0.63

Abbreviations: CMV, cytomegalovirus; M, million; WGS, whole-genome sequencing; X, X-fold.

SNP-based techniques rely on informative SNP loci to separate donor and recipient genotype. The theoretical number of informative SNPs for a particular assay is Npq, where "N" equals the total number of SNP in the panel, and "p" and "q" represent the average frequency of major and minor allele in the population, respectively. The maximum number of informative SNPs is 0.25 N, when $P = q = 0.5$. The ratio of homozygous and heterozygous informative SNPs (AA + Aa and AA + aa) will also impact the quantification result of ddcfDNA level, and it fluctuates easily if the total number of informative SNP is small. CareDx, Inc, the manufacturer of the AlloMap peripheral gene expression assay, has recently developed the AlloSure cell-free DNA assay (CareDx Inc, Brisbane, Calif, United States) for transplant rejection surveillance that uses targeted amplification of 266 SNPs to reliably distinguish donor from recipient cell-free DNA [3]. Comparing to AlloSure, our study applied a total of 6200 SNPs for the measurement of ddcfDNA concentration, and the identified 1000 to 1500 informative SNPs had a relatively stable combination of AA + Aa/AA + aa for a certain population. In addition, capture probes of 2000 microorganisms in one kit make it capable of screening to pathogens.

The ddcfDNA concentrations were significantly different among stable recipients after liver [26], kidney [24], lung [27], and heart transplantation [28], which indicates that various thresholds of ddcfDNA concentration implying acute rejection should be applied for different types of organ transplantations in determining acute rejection. Moreira et al [13] showed early increases of total cfDNA levels during acute rejection, systemic infection, and acute tubular necrosis compared to patients with stable graft function after kidney transplantation. Recently, Bloom et al [25] reported that a ddcfDNA level above 1% might indicate active rejection in a kidney allograft, and ddcfDNA is a better biomarker for indicating rejection compared to creatinine. Here, our result indicated that the ddcfDNA levels in patients with acute rejection (1.87%) were revealed to be significantly higher than that in the comparator group (0.90%) of biopsy specimens without active rejection ($P < .001$).

Donor-derived infections could include viruses [29], bacterial [30,31], and several fungal organisms after kidney transplantation. Among these, virus infection has emerged as a major complication. CMV and BK virus are most frequently assayed clinically. Plasma ddcfDNA levels have

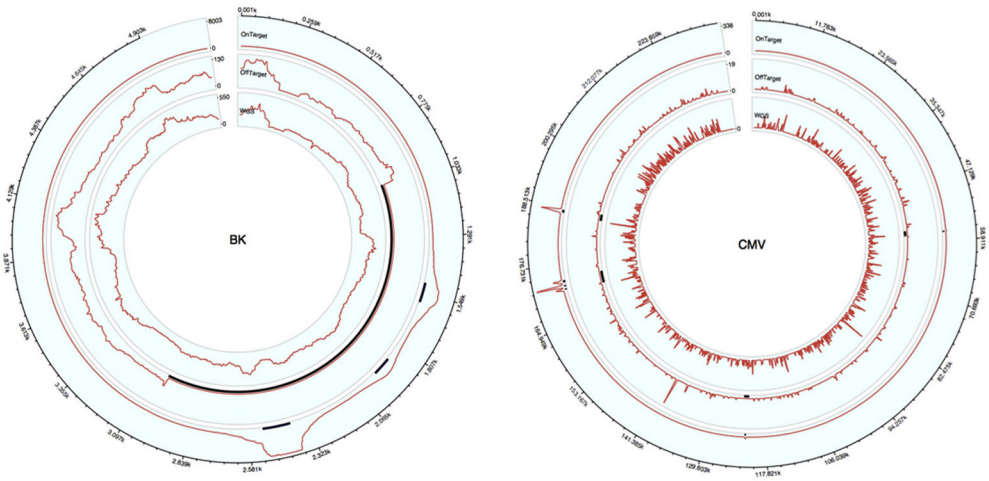

Fig 3. Sequencing depth distribution across virus genome for BK virus–infected samples (left, 5.15 Kb) and cytomegalovirus (CMV)-infected samples (right, 234.64 Kb). The inner and middle circle was plotted using whole-genome sequencing (WGS) and off-target reads, respectively. The distribution of outer circle included the whole-targeted sequencing data, regardless of on-target or off-target reads. Middle circles marked by black curve present the targeted area and 500-bp flanking regions. Probe sequences are indicated by black curves on the outer circle.

shown marked increases both during acute rejection and graft infection, pointing to the necessity of a combined pathogen monitoring strategy [10]. Here, the levels of CMV-derived and BK virus–derived sequences in cfDNA were consistent with clinical results.

Conventional methods for detecting pathogens such as PCR, real-time multiplex polymerase are useful tools for the detection of microorganisms that are difficult to cultivate and those that grow slowly, which only allow the identification of known pathogens with limited throughput [32]. Recent techniques applying NGS make it possible to screen a large number of pathogens at one time [33,34]. With this technique, targeted 2000 pathogenic microorganisms can be detected by sequencing the species-specific genome region except for BK virus and parvovirus. It is worth noting that a substantial number of viral sequences were identified across the whole viral genome, indicating that the off-target sequencing reads are similar to WGS methods. Off-target sequences introduced were mainly due to the insufficient blocking of adaptor sequences during targeted area hybridization and capture. Those reads were usually undesired and treated as wasted data, however, our work indicates that the sequences from off-target areas can be used for further confirmation of the presence of pathogen infection.

The assay presented herein has the potential to become an important tool for transplant surveillance, considering 1. the high incidence of acute rejection and difficult-to-diagnose infectious complications; 2. the numerous limitations of biopsy in rejection surveillance; 3. posttransplantation infection and rejection often present with similar symptoms; and 4. standard clinical tests often fail to distinguish between these 2 common complications [2]. It should, however, be noted that there are several limitations in our paper. First, the limited sample size, especially when considering subset comparisons, does not have adequate statistical power to detect T cell–mediated rejection and antibody-mediated rejection. Second, subclasses of rejection observed among these biopsy specimens was limited. Thirdly, clinically positive sample size by pathogen infection was limited. However, our data mainly revealed that the noninvasive and donor-independent method can simultaneously monitor rejection and infection in patients with history of organ transplant.

Moreover, the turn-around time required for executing the process was approximately 3 days including cfDNA extraction, library construction, capture SNPs, and sequencing on the Illumina NextSeq 500 platform. The cost involved for testing samples was approximately $1500; this cost can be lowered and made available to all patients as the technique becomes more efficient thus requiring less time and resources. In terms of logistics, the 4 mL to 8 mL of peripheral blood needed for the sample from patients with kidney transplants can be adequately obtained using a cfDNA blood collection tube. The peripheral blood in the cfDNA blood collection tube was transported at room temperature. Aside peripheral blood, urine samples from patients with a history of kidney transplant could also be used to conduct a cfDNA investigation, which happens to be our next research target.

In summary, our study established a noninvasive methodology to simultaneously monitor rejection and infection in patients with allograft transplants. Immunosuppression therapy is widely adopted by patients with allograft transplants to reduce the risk of transplanted rejection, however, a compromised immune system increases the patient's risk of being infected by pathogenic microorganisms. By monitoring both rejection and infection for patients with a history of allograft transplantation, this technique provides comprehensive information for clinicians to optimize the personalized immunosuppression therapy for the patients.

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