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Rapid detection of donor cell free DNA in lung transplant recipients with rejections using donor-recipient HLA mismatch ☆

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ABSTRACT

Fiberoptic bronchoscopy and transbronchial lung biopsy are currently the gold standard for detection of acute rejection following human lung transplantation (LTx). However, these surveillance procedures are expensive and invasive. Up to now, there are few new methods that have demonstrated clinical utility for detecting early stages of rejection following human lung transplantation. We optimized and technically validated a novel method to quantify donor-derived circulating cell free DNA (DcfDNA) that can be used as an early biomarker for lung allograft rejection. The method involves the initial development of a panel of probes in which each probe will specifically target a unique sequence of a human leukocyte antigen (HLA) allele. After transplantation, donor/recipient specific probes are chosen based on the mismatched HLA loci, followed by droplet digital PCR (ddPCR) used as a quantitative assay to accurately track the trace amount of DcfDNA in an ample excess of recipient DNA background. The average false positive rate noted was about 1 per 800,000 molecules. Serially 2-fold diluted cfDNA, representing donor fractions of cfDNA, were spiked into a constant level of cfDNA representing the recipient cfDNA. The fraction of spiked cfDNA was measured and quantitative linearity was observed across seven serially diluted cfDNA samples. We were able to measure the minor portion of cfDNA as low as 0.2% of total cfDNA. We subsequently applied the method to a pilot set of 18 LTx recipients grouped into biopsy-proven acute rejection, bronchiolitis obliterans syndrome (BOS) or stable groups. Serial plasma samples were used to identify the percentage of DcfDNA over total cfDNA. The level of DcfDNA was significantly elevated in patients diagnosed with acute rejection (10.30 ± 2.80, $n = 18$), compared to that from stable (1.71 ± 0.50, $n = 24$) or from BOS patients (2.52 ± 0.62, $n = 20$). In conclusion, we present results validating the application of digital PCR to quantify DcfDNA assay in primary clinical specimens, which demonstrate that DcfDNA can be used as an early non-invasive biomarker for acute lung allograft rejection.
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1. Introduction

Lung transplantation (LTx) is the accepted treatment for advanced lung disease. The United Network for Organ Sharing (UNOS) reported 2057 lung transplants in the US in 2015 (www.unos.org/about/annual-report). Despite recent advances in medical management of LTx, the median 5-year survival of recipients remains the lowest among the major solid organ allografts [1]. Currently, fiberoptic bronchoscopy and transbronchial lung biopsy is

Abbreviations: BAL, broncheoalveolar lavage; BOS, bronchiolitis obliterans syndrome; DcfDNA, donor cell free DNA; ddPCR, droplet digital PCR; HLA, human leucocyte antigen; LTx, lung transplantation; LTxR, lung transplantation recipient; qPCR, quantitative PCR; SNP, single-nucleotide polymorphism; TBB, transbronchial biopsy.

☆ JZ, BD, and TM participated in research design; JZ, BD, NS and AY participated in performance of the research; JZ, BD, RH and MS participated in data analysis and the writing of the paper.

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the gold standard for detection of allograft dysfunction post LTx [2]. However, this surveillance procedure is expensive and invasive. Scott et al. studied the sensitivity and specificity of transbronchial biopsy (TBB) in heart-lung and single LTx recipients and found eighteen samples per procedure were needed to reach 95% confidence of rejection detection [3]. In light of these limitations on the use of biopsy, new methods that aim to detect early rejection are needed. However, few studies have definitively demonstrated routine clinical utility at early clinical stages of presentation, since most methods rely on relatively non-specific and/or insensitive biomarkers [4].

Circulating cell free DNA, which is likely derived from apoptotic tissue or cells, is now considered as "liquid biopsy" in early detection of cancer and monitoring therapeutic effects [5]. Following allogeneic organ transplantation, DcfDNA has potential as a biomarker of graft dysfunction or rejection following renal and pancreas transplantation [6,7]. Persistent increases in DcfDNA were detected in pancreas-kidney transplantation patients with biopsy-confirmed rejection [8]. It has been also reported that levels of DcfDNA reduced significantly (from 66% to <5%) when tacrolimus concentration was adjusted from under-therapeutic level to therapeutic level following liver transplantation [9], suggesting that DcfDNA can also be used to adjust the immunosuppression. Reports from Dr. Quake's lab following human heart transplantation suggested that the percentage of DcfDNA was less than 1% at stable patients, whereas the level can increase up to 5% during a rejection episode [10,11]. A recent report has shown that DcfDNA can be used in detection of rejection in LTx as well [12]. In their studies, single-nucleotide polymorphism (SNPs) markers were employed using shotgun sequencing to discriminate donor and recipient derived cfDNA. This approach however can be costly and time consuming. In addition, obtaining the donor DNA post-transplantation may not always be feasible. However, HLA phenotypic variation between donor and recipient are commonly seen in all of the solid organ transplantations. For instance, over 85% of LTx has at least 4 out 6 mismatched HLA alleles, and less than 0.1% of LTx are 6 out 6 matched [13]. The information of mismatched HLA alleles is available even prior to the transplantation since HLA typing from donor and recipients are a part of routine transplantation workup in most centers.

For this study, we developed a novel, rapid, cost-effective, genomics-based approach based on variation between mismatched HLA alleles. We optimized and technically validated this method to quantify DcfDNA that can be used as personalized markers for rejection. The method involves the initial development of a probe panel in which each probe will specifically target a unique sequence on HLA alleles. After transplantation, donor/recipient specific probes are chosen based on the mismatched HLA loci, followed by ddPCR used as a quantitative assay to track the trace amount of DcfDNA in an extensive excess of recipient DNA background. Additionally, we applied the method to a pilot set of 18 LTx patients to test the clinical applicability**.** We demonstrate that this novel validated assay can be applied to routine clinical specimens for the detection of early graft rejection following human LTx using individual HLA variants making this as a non-invasive biomarker for acute rejection following solid organ transplantations.

2. Methods and materials

2.1. HLA-specific primers and probes

A semi-personalized panel of HLA-specific targeting primers and probes were designed and synthesized. Two pairs of primers flanking two regions containing eight of the most common HLA-DR alleles, including HLA-DRB1∗01, HLA-DRB1∗03/DRB3∗01, HLA-DRB1∗04, HLA-DRB1∗07 HLA-DRB1∗08 HLA-DRB1∗11 HLA-DRB1∗13 HLA-DRB1∗15/∗16 were synthesized (Fig. 1). Produced amplicon sizes were designed to be less than 70 bp giving that circulating cfDNA is largely fragmented [14]. Additionally, eight probes targeting each allele specifically were generated (Red boxes Fig. 1). Each probe was generated in two versions containing either a HEX or a FAM fluorophore and they are now commercially available at BioRad (ID: dHsaEXD29156242, dHsaEXD93426015, dHsaEXD67695788, dHsaEXD41965561, dHsaEXD16235334, dHsaEXD80505107, dHsaEXD54774880, dHsaEXD29044653). The DcfDNA and recipient cfDNA give signals in different channels, allowing the ratio between donor and recipient cfDNA to be calculated.

2.2. Patients selection

Sixty-two serum samples at multiple time points from 18 adult bilateral LTx recipients (LTxR) were selected and subjected to cfDNA extraction and measurement, respectively. All transplantations were performed at Barnes-Jewish Hospital (St. Louis, MO) between 2002 and 2014. Twenty-four samples from 7 stable LTxR, 20 samples from 5 LTxR diagnosed with Bronchiolitis Obliterans Syndrome (BOS) and 18 samples from 6 LTxR with biopsy-proven acute rejection were included in the study. We excluded the samples collected within two weeks post-transplantation, as it has been shown that the donor contributed cfDNA was elevated for a short period of time after transplantation probably due to ischemia-reperfusion injury [12]. All of the LTxR underwent surveillance bronchoscopy with broncheoalveolar lavage (BAL) collection and TBB during episodes of clinical deterioration. The allograft status was determined based on microbiologic cultures, radiographic studies and biopsy notes. Acute and chronic rejection was defined by pathologic diagnosis on TBB and graded according to the standard International Society for Heart and Lung Transplantation (ISHLT) criteria [15]. The HLA typing for the donors and recipients were performed at the HLA laboratory at Barnes-Jewish Hospital. Informed consent was obtained on all LTxR and was approved by the human studies institutional review board (IRB#201103312) Washington University Human Studies Committee.

2.3. Isolation of cfDNA and pre-amplification

The cfDNA isolation protocol was modified from a well-established procedure [9,10]. Briefly, the plasma was separated from peripheral blood cells within four hours of sample collection by centrifugation at 1600*g* for 10 min. The serum was stored at −80 °C until DNA extraction. cfDNA was extracted from 2 ml aliquots (of patient plasma using the QIAamp circulating nucleic acid kit (Qiagen) according to the manufacturer's instructions. To increase the total number of DNA molecules tested, isolated cfDNA was amplified by utilizing an unbiased pre-amplification method from Bio-Rad (SSo PreAmp Assays). Each 50ul reaction included: 25 ul of SSoAdvanced PreAmp Supermix; 12ul of PreAmp assay pool (BioRad commercial ID dHsaEXD93314426 for region 1 and dHsaEXD67584199 for region 2); 12 ul of water and 8ul of isolated cfDNA. The PCR was conducted as: 95 °C for 3 min, 10× (95 °C for 15 s, 58 °C for 4 min). 2 μl of the product was used in the following Droplet digital PCR assay.

2.4. Droplet digital PCR (ddpcR)

Corresponding probes were selected based on the mismatched HLA-DR alleles (Table 1). The ddPCR was set up according to manufacturer instructions (Bio-Rad). The Bio-Rad QX200 ddPCR system was used, which partitions each sample and PCR assay mixture,

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