A noninvasive assay for monitoring renal allograft status

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Abstract Transplant rejection is a serious complication, sometimes threatening life of the patient. Although recent development of the new generation of immunosuppressive drugs reduced the incidence of acute rejection in kidney transplantation, the absence of noninvasive biomarkers of the rejection does not allow often the optimization of a prompt antirejection therapy. Serum creatinine is the most widely used marker for allograft function, however, it is not sensitive and specific enough to detect acute rejection. Other biomarkers are even less valuable for this purpose. Histological examination of renal allograft biopsy still remains the golden standard for diagnosing acute renal allograft rejection. Therefore, there is a high demand for reliable biomarkers for noninvasive monitoring of renal allograft status. Examination of urine in renal transplant recipients provides a logical and readily accessible approach for this monitoring. The high potency biomarkers for kidney allograft monitoring are fragments of DNA in recipient urine that originated from renal allograft cells. Because of the difference in the genetic origin these DNA can be distinguished from recipient DNA. Quantitative analysis of donor's DNA, derived from cells of renal allograft, in recipient's urine might be a reliable predictive tool for the kidney transplant rejection. We developed an assay to quantitate donor DNA content in recipient urine. Application of the technique—coamplification at lower denaturation temperature-PCR (COLD-PCR) increased the abundance of donor DNA that usually presents in recipient urine in quantities that are out of the detection range. This assay has a potential for routine application in clinical practice after statistical validation and additional modifications.

Keywords: renal allograft, acute rejection, COLD-PCR, cell-free DNA

INTRODUCTION

Kidney transplantation is the treatment of choice for most patients with end-stage renal disease. About 15,000 kidney transplantations are performed per year in USA and assays for monitoring of allograft status are highly demanded. Acute rejection is an important risk factor for allograft failure [1]. Despite the dramatic improvement in immunological matching, surgical techniques and, especially, in the development of new immunosuppressive drugs, acute rejection is still a serious medical problem among kidney transplant recipients (6-10%). Early diagnosis of acute allograft rejection is critical for managing the therapeutic approach and for long-term surviving of the transplanted kidney [2].

Typically, renal allograft function is evaluated by measuring serum creatinine (SC) levels. Unfortunately, SC level elevation is a non-specific marker of renal allograft dysfunction, and it may occur in many different conditions. Currently, kidney allograft biopsy remains the "gold standard" for assessing the cause of kidney transplant dysfunction. However, this procedure is damaging, invasive and expensive [3]. The alternative for the surgical intervention is a search for novel noninvasive biomarkers that will be able to substitute the biopsy, predict clinical outcomes, to determine the treatment starting point and regimen and, as a result, will improve allograft survival.

Examination of the urine in renal transplant recipients provides a logical and readily accessible non-invasive approach on allograft function in its transplanted environment. Renal tubular epithelial cells (TEC) respond dynamically to the surrounding microenvironment and play an important role in allograft survival. Proteins and DNA released from TEC into the urine potentially can serve as biomarkers for the early diagnosis of graft dysfunction and rejection. Due to the proximity of TEC to the tubular lumen, these proteins, DNA and other cell components are passed directly into the urine [4]. Proteomics and genomics have been utilized to search for new urine biomarkers for early diagnostics and treatment of kidney allograft rejection. Several urinary biomarkers (mRNA, miRNA and protein) and especially their combination showed some promise in diagnosis of acute renal rejection [2, 3, 5]. However, there are limitations in expanding the application of these biomarkers from research to clinical practice based on the limited statistically relevant data and on the expensive search and complicated candidate validation techniques.

The highly promising alternative to the monitoring specific individual biomarkers is a cell-free donor DNA in the urine of the transplant recipient. It has been shown since the 1950s that ng/ml amounts of DNA can be detected in human urine [3].

Since the circulating cell-free donor's DNA in urine of a recipient is genetically different from the recipient DNA it might be used for molecular diagnostics of renal transplant status. To distinguish the donor's DNA in urine from DNA derived from host cells it is necessary to determine person-specific differences in the sequences of their genomic DNA. Several approaches have been already proposed to achieve this task. The novel form of chimerism, termed urinary DNA chimerism, has been described in kidney transplant recipients. Highly polymorphic and individually specific short tandem repeat (STR) loci, were used in

this study to detect donor cell-free DNA in recipient urine. Quantitative analysis of this DNA in urine has indicated that it may serve as a new marker for monitoring kidney transplant status [6]. Another example of using urine donor's DNA as a biomarker for allograft status is the detection of donor-specific DNA polymorphism in the urine of the recipient in sex–disparate kidney donor-recipient conditions [7-9]. These approaches are restricted by the limited type of donor-recipient pairs.

Recently the more universal tactic, applicable for any combinations of donor-recipient pairs, relied on the new molecular genetic technologies: Next Generation Exome Sequencing has been introduced [10]. In this study, allograft status was monitored by measuring the amount of donor cell-free DNA circulating in the blood of heart transplant recipients. Statistically significant increase of donor's cell-free DNA was observed at times when an endomyocardial biopsy independently established the presence of acute cellular rejection in heart transplant recipients [10]. Although this approach is logical, straightforward and universal, it is very expensive, time–consuming and demands special, not easily achievable yet, Next Generation Sequencing platforms, software for bioinformatics analysis and highly qualified, specially trained personnel.

The measurement of donor's DNA in recipient urine is a logical approach for renal allograft monitoring. Although it has been shown that the amount of donor cell free DNA in recipient urine reflects the renal allograft status, the limited statistical significance of these studies, insufficient sensitivity of the assays and/or complexity of the approaches may hinder the transfer of these techniques to the clinic.

We propose herein a simple, cost-effective procedure for quantitative analysis of donor's DNA content in the recipient urine, based on the predetermined genetic differences in the mitochondrial DNA (mtDNA) between the donor and the recipient. Analysis of mtDNA was selected because of the known high concentration of single nucleotide polymorphism (SNP) in the relatively small area of mtDNA-D-loop [11], allowing convenient amplification and sequence analysis. The sequence variance in this region for 2 random subjects allows one to distinguish mtDNA of donor and recipient mixed in recipient urine. This analysis will be applied for monitoring the clinical status of renal allograft.

The quantitative analysis of donor's DNA, originated from dying cells of renal allograft in recipient urine, will be a reliable predictive tool for kidney transplant rejection. The technique includes isolation of DNA from urine, modified PCR assays and pyro or Sanger sequencing, substituting more expensive and complicated techniques such as Next Generation Sequencing, ELISA, qPCR or Luminex. Bioinformatics and statistical analysis of these data will be effective and straightforward and will not demand special software platforms.

MATERIALS AND METHODS

Detailed description of the suggested analysis is presented in Figure 1.

1. Identification of genetic differences by sequencing of mtDNA

To determine the differences in genomic DNA of two random individuals (donor and recipient) we compared sequences of highly variable locus of D-loop in their mitochondrial DNA, which contains more than 100 SNPs. Based on the results of forensic research, the probability of perfect match for these sequences for 2 non-related subjects is low (0.5-0.8%) [11]. Consequently, the difference in these sequences (SNPs) can be used to distinguish donor and recipient DNA in the urine of a renal allograft recipient for the most random pairs. Genomic DNA was obtained from related donor and recipient (provided by Tissue Type laboratory). All patients gave informed consent to the protocol approved

by our institutional review board for studies in human subjects. PCR amplification of HVR1 and HVR2 region of mtDNA was performed with BioRad MJ Mini Thermocycler (BioRad, Hercules, CA) using Fast Cycling PCR kit cat# 203741 (Qiagen, Inc Valencia CA) and related primers [12]. Primers were ordered from Eurofins MWG Operon (Huntsville, AL). Sequencing was performed with forward PCR primer. Sequences were aligned using BLAST software and SNPs that are different for donor and recipient were determined.

2. Isolation of cell-free DNA

DNA from 10 ml of urine was isolated using the Urine DNA isolation micro kit (cat# 48800, Norgen Biotek. Corp, Ontario, Canada). Concentration of isolated DNA was determined using NanoDrop (Thermo Scientific, DE). For every assayed sample we have shown that donor's mtDNA is indeed present in the urine of the renal allograft recipient. However, the abundance of donor's DNA in urine is low. If the total cell-free DNA concentration in urine is ranged from 20 to 1000 ng / ml, the donor's DNA may be present as 0.1% of the total amount (pg/ml). This concentration is out of the detection range for conventional detection techniques even after PCR amplification.
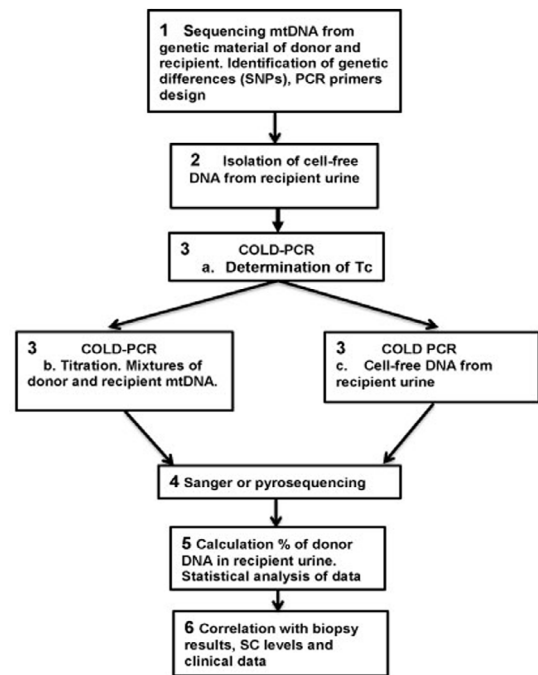

Figure 1. Schema of the proposed procedure.

3. COLD-PCR

To increase the proportion of a low abundant allele we have used a recently developed technique: COLD-PCR that enables selective amplifications of low abundant DNA variants from mixtures of wild-type and mutant-containing sequences irrespective of the mutation type or position on the amplicon, by using a critical denaturation temperature (Tc) [13, 14]. The general outlines of the procedure are described in several articles and reviews [13, 14] COLD-PCR can be applied in several formats: full-COLD-PCR enriches all types of mutations along the sequence, however enrichment is modest (3-10 folds); fast-COLD-PCR results to enrichments of 10- to 100-fold, and is robust and time-efficient, but is limited to enriching only Tm-reducing mutations (G:C>A:T or G:C>T:A [15]. More recently developed platform incorporates a synthetic reference sequence within a PCR, designed to enhance amplification

of unknown mutant sequences during COLD-PCR. This new platform enables an Improved and Complete Enrichment (ice-COLD-PCR) for all mutation types and eliminates shortcomings of previous formats of COLD-PCR (full- and fast COLD-PCR [15]. Another version of COLD-PCR enables simultaneous enrichment of mutations in several amplicons and increases significantly the versatility of COLD-PC [16].

For the current study we used Full COLD-PCR that is most applicable for any type of mutations. Full COLD-PCR uses a hybridization step at an intermediate temperature during PCR cycling to allow cross-hybridization of mutant and wild-type alleles. Heteroduplexes, which melt at lower temperatures than homoduplexes, are subsequently selectively denatured at the Tc and amplified throughout the course of the PCR [17].

3a. Determination of critical temperature. The first and highly important step in the design of the COLD–PCR reaction is determination of critical temperature (Tc). The Tc is lower than standard denaturation temperatures in that it preferentially denatures heteroduplexed molecules (those formed by hybridization of mutant and wild-type sequences) and amplicons possessing mutations that lower the amplicon melting temperature (Tm), such as G:C>A:T or G:C>T:A [16].

The original presentation of the method suggests several ways to determine this parameter: post-PCR real time melting curves analysis or theoretical calculation using DNA melting software such as MeltSim [18]. To simplify the procedure for the future possible clinical application we compared several ways of Tc determination and concluded that MeltSim software calculates melting temperature of amplicon Tm (the parameter for Tc determination) with high accuracy that is adequate to our application [13, 19]. Moreover, to further simplify the procedure for clinical application we determined that the same Tc can be used in other experimental settings. Minor adjustment might be needed for the cases with highly abundant mutations. DNA products amplified by COLD-PCR can be directly sequenced using Sanger or pyrosequencing.

3b. Preparation of template DNA. Mixtures of donor/recipient mtDNA (1, 2, 5, 10, 20%) will be used as templates for COLD-PCR reaction with selected primers and predetermined Tc.

3c. Data collection. Cell-free DNA from patients (recipient) urine at different time points will be used for COLD-PCR reactions. Urine will be collected during transplant patient visit clinic every 1-2 weeks after the surgery and in the hospital if patient will need hospitalization. In addition urine will be collected before and after biopsy as often as it will be possible. At the same time points SC data will be recorded.

4. Sequencing

Sanger sequencing currently is the most common procedure for determination DNA sequence. Pyrosequencing is an alternative to the Sanger sequencing. This method provides several advantages for the proposed application; in particular: a. Amplicons after COLD-PCR reactions are of the size that is most applicable for pyrosequencing, b. Pyrosequencing is much faster procedure than Sanger sequencing (results are available in 2 hours) c. Pyrosequencing is cost-effective. d. Pyrosequencing software allows direct calculation of the proportion of 2 alleles in the mixture without measuring peak amplitudes. e. The bias of this method also can be less than for Sanger sequencing because in this method fluorescent-labeled dNTPs are not used. Pyrosequencing of COLD-PCR amplicons provides consistent data. However this type of sequencing needs more preparative work (primer design). An example of pyrosequencing results for COLD-PCR amplicon of cell free DNA isolated from urine of transplant recipient is presented in **Figure 2**.
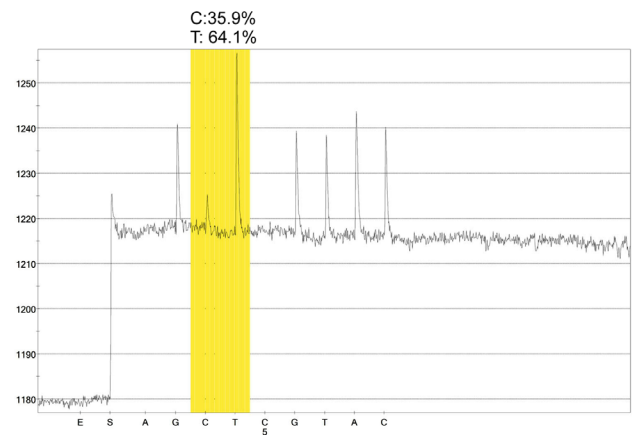

Figure 2. Pyrosequencing diagram of DNA purified from urine of renal transplant patient. Proportion of donor DNA (allele with C nucleotide- 35%, with T-65%) is shown. Y-axis: light intensity (relative units).

5. Statistical analysis

The calculation of the original concentration of low abundant allele in recipient urine based on the analysis of sequencing data for COLD-PCR amplicons is not a straight-forward task. Two major sequencing techniques are utilized: Sanger or pyrosequencing (step 4 in **Fig. 1**). When Sanger sequencing is performed, the primary bias source is the procedure itself. When the simple approach by measuring fluorescence intensity peaks on the sequencing chromatogram was applied for the allele frequency calculation of a heterozygote, the expected frequency 50% was not observed [19]. Possible reasons are different fluorescence intensities and the incorporation efficiency for distinct fluorescence-labeled dNTPs. In this study the authors solved the problem by including heterozygote in the analysis and by using the ratio of two allele-specific peak heights of the heterozygote (k) for the correction of the estimated mutant frequency. This correction allowed more accurate calculation of original mutant concentration in the mixture using modified Equation 1 [19]. The relative content of donor and recipient DNA is calculated by the measuring amplitude of peaks for mutated nucleotide on sequencing chromatogram for both alleles.

$$Fd = \frac{Hd}{Hd + k * Hr} * 100\%$$

Equation 1. Modifications for calculation % of donor DNA in urine. *Fd*-donor allele frequency, *Hd*-height of the peak of mutated allele, *Hr*-height of the peak of wild allele

We adjusted this equation for our needs. In the current study we utilized mtDNA, therefore, instead of using heterozygote for the correction of the errors, we performed conventional PCR reaction with the same primers as for COLD-PCR and as a template the mixture of 50% recipient/50% donor mtDNA. The ratio of the two peaks on the sequencing chromatogram (k) will be used for the correction of the calculations in the modified Equation 1.

Another barrier to the calculation of the original concentration of low abundant allele in recipient urine based on the analysis of sequencing data of COLD-PCR amplicons is the nature of the COLD-PCR reaction since amplification efficiency of low abundant allele by COLD-PCR depends of the original concentration of the minor allele in the assay mixture and of the chemical composition of the amplicon [20].

To establish a reliable procedure for calculation the original amount (before amplification) of donor DNA in the recipient urine we first

performed COLD-PCR-sequencing analysis of serial dilution of donor / recipient DNA. An example of the COLD-PCR amplification-sequencing results from serially diluted donor/recipient DNA and calibration curve based on these data is presented in **Figure 3A**.
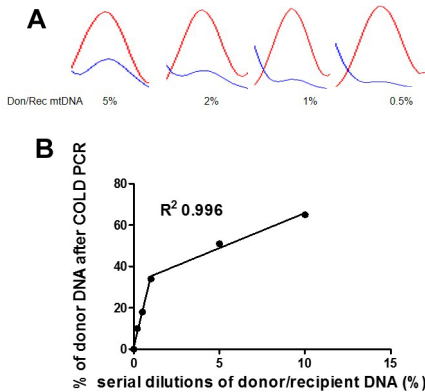
Figure 3. Calibration curve and regression analysis for correction of donor DNA measurement in recipient urine. A. Sequencing chromatograms of mtDNA mixtures after COLD-PCR reaction. Proportion of donor's DNA is calculated based on the measure of chromatographic peaks amplitudes. Blue curve (C)- donor, red curve (T) -recipient. **B.** Calibration plot and segmental regression analysis.

The results of these calculations are plotted (**Fig. 3B**). On the Y-coordinate is % of donor DNA calculated by measuring peaks amplitudes after COLD-PCR and sequencing and corrections using Equation 1. To use this plot as a calibration curve for the calculation of real concentration of donor's DNA in recipient urine we first applied simple linear regression analysis. This type of analysis was not adequate for the current application. However, segmental linear regression analysis provides the basis for the reliable calculation of % of DNA in urine based on the sequencing COLD-PCR amplicons using this plot as a calibration curve. Segmental regression fits one line to all data points with X less than some value $X0$, and another line to all points with X greater than $X0$, ensuring that the two lines intersect at $X0$. An example of such plot and application of segmental regression analysis is presented in **Figure 3B**. This calibration process is planned for every donor/recipient pair. However, since amplicons often are substantially similar for different donor-recipient pairs, it is possible that the same calibration curve can be used in the future for amplicons with similar type of mutations.

6. Correlation with biopsy results, SC levels and clinical data

The relative value of donor/recipient cell-free DNA content in the recipient urine measured and after correction with calibration curve will be plotted over time after transplantation. SC level will be also monitored during this period. This factor is routinely assayed for all transplant patients. The decision about treatment or renal biopsy is generally made based on the elevation of SC over base line for 10-25%. Therefore, we suggest that donor DNA elevation in the urine can precede SC increase, and can represents a more accurate sign of the potential allograft rejection. Since the clinical picture is usually complicated, we plan to collect substantial amount of the data and to use comprehensive statistical analysis for data interpretation. SAS statistical software will be utilized for this analysis. The relationship between urine donor's DNA and SC will be assessed by analysis of variance. A conventional receiver operating characteristic (ROC) will be generated for urine donor DNA at the point when it will be increased by 25% to the baseline. The area under the curve will be calculated to determine the quality of

donor DNA as a predictive biomarker for acute rejection. Area of value 1 will be considered as a perfect biomarker. Positive biopsy will be an indicator of true positive. Clinical application statistical analysis will be simplified and adjusted for the use with readily available software.

RESULTS

We collected urine from patients that underwent renal transplantation, analyzed cell free DNA in these samples and developed the assay for quantitation of donor/recipient DNA ratio in urine. Urine was collected during every visit of transplant clinic after the surgery. Cell-free DNA from urine was isolated and kept frozen, ready for COLD-PCR and sequencing. SC data for these time points was documented. SC level is usually used as a surrogate marker for allograft status including prediction of acute rejection. When SC level increased over 10% we performed COLD-PCR and sequencing analysis of the urine samples collected for these patients. Once biopsy to examine the allograft status was scheduled we obtained all available data (donor DNA level in urine) for this patient. Based on these results we can conclude: 1). Donor mtDNA can be detected in urine of recipient. 2) This detection is possible even for the minimal amount of donor DNA in urine (0.2%) by using a modified COLD-PCR technique. 3) The level of donor DNA in urine of renal transplant recipients is a dynamic parameter.

To explore the possibility of using this parameter for the prediction of renal acute rejection we analyzed the kinetics of donor DNA level in urine of patients that were scheduled for biopsy because of the increase of SC level. One patient was under our observation during 27 weeks and was scheduled twice for biopsy; at week 7 and 22. Both times the biopsy confirmed acute renal rejection. Another patient was 14 weeks under our observation and was scheduled for biopsy at week 5 after transplantation. This biopsy did not confirm acute renal rejection (**Fig. 4**). The time course of donor DNA % of total cell–free DNA in recipient urine and SC level for the same time points is presented in **Figure 4**.
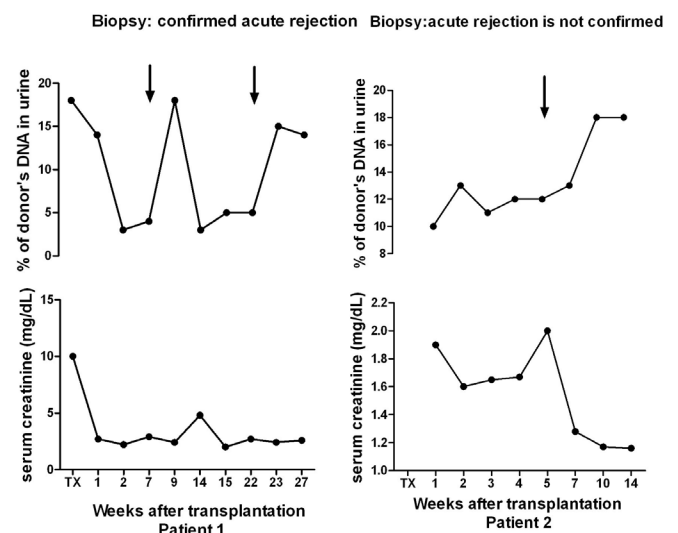
Figure 4. Time course graphs for % of donor DNA level in urine and SC for the same patients. Patient 1 (left panel) had 2 biopsy-confirmed acute cellular rejections (indicated by arrows). Patient 2 (right panel) had 1 biopsy that did not show acute rejection.

Analysis of these graphs shows that for Patient 1, after the second week post-transplantation, SC increased by 30%, which was an indication of possible rejection. Donor DNA in patient's urine was also increased by 30-35% during which the first biopsy was performed at

week 7. After the biopsy level of SC returned to normal, donor DNA in the urine continued to increase, possibly because of the allograft damage during the biopsy. However, the kinetics of donor DNA level in recipient urine not always coincided with the kinetics of SC level. Indication for another possible rejection was after week 15 when SC levels was increased about 30% and acute rejection was confirmed at a later time by biopsy. However, the level of donor DNA in this case started rising at week 14, suggesting that this marker can be used for earlier prediction of allograft rejection. Patient 2 had an allograft biopsy due to a 25% increase in SC after week 4. In this case, biopsy did not show acute allograft rejection and donor DNA elevation was not detected during the same time period. Therefore, donor DNA content in urine may be a more accurate marker for prediction of acute rejection.

DISCUSSION

In this study we aimed to demonstrate that donor-derived cell-free DNA is present in the urine of kidney transplant recipients, and that elevated levels of donor DNA can be used as an indication of allograft rejection. Analysis of donor DNA in urine may be a more accurate marker than those that are currently used. Our data establish unambiguously that donor-specific DNA is present in the urine and can be accurately quantitated. This quantitation during the time after transplantation allows the evaluation of donor DNA content as a noninvasive marker for monitoring renal allograft status.

Currently the assay includes: DNA isolation from genetic material of donor and recipient, sequencing and comparison of the target sequences. This is a routine procedure for any clinical genetic study and can be easily automated. Isolation procedure is reliable and can be automated, possible in collaboration with Norgen Biotek, Corp. that manufactures urine DNA isolation kits for clinical applications. COLD-PCR amplification of cell-free DNA from urine is a relatively simple and reliable procedure. As soon as SNPs in D-loop specific for donor and recipient are determined COLD-PCR can be performed in any clinical laboratory. Quantitative analysis of donor DNA includes several simple steps exploiting widely used software. COLD-PCR amplicons can be sequenced using primers that were designed for amplification. All devices that are necessary for this assay are common in genetically oriented clinical laboratories. Sequencing service is also highly available.

The current study can be considered as a proof of principle that elevated level of donor-derived DNA in urine may be a better predictive marker for allograft functions. Additional studies will allow us to define the feasibility of the assay and to determine how often urine analysis of cell-free DNA must be performed. Considering that the most renal allograft rejection events occurred during first 6 month after the transplantation, 6-40 urine samples from the patient will be necessary. Genetic material for the primary sequencing is already available for each pair of donor-recipient. Despite the dramatic improvement in renal transplant technology, potential complications must be monitored and addressed in a timely fashion.

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