Use of Graft-Derived Cell-Free DNA as an Organ Integrity Biomarker to Reexamine Effective Tacrolimus Trough Concentrations After Liver Transplantation

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Background: Immunosuppressant therapeutic ranges for transplant patients have traditionally been established by indirect clinical means. However, “liquid biopsy” methods measuring graft-derived cell-free DNA (GcfDNA) in blood directly interrogate donor organ integrity. This study was performed to determine whether GcfDNA quantification could be used to reexamine minimally effective trough tacrolimus (Tacro) concentrations in liver transplantation (LTx) patients.

Methods: As part of a large prospective study to demonstrate the ability of GcfDNA to identify early graft rejection, 10 adult white LTx patients [8 men, 2 women, 3 hepatitis C virus (HCV) positive; mean ± SD age (years) = 56 ± 9.4] had simultaneous GcfDNA and whole-blood trough Tacro concentrations measured between days 5 and 30 after LTx. Samples were analyzed using droplet digital polymerase chain reaction for GcfDNA and liquid chromatography tandem mass spectrometry for Tacro. GcfDNA and trough Tacro concentrations were then compared to identify Tacro concentrations associated with intact graft integrity.

Results: Although there were large individual differences, there was a highly significant (Fisher P = 0.00002) segregation between whole-blood Tacro concentrations of ≥8 μg/L and normal (<10%) GcfDNA percentages. The best discrimination in this population between effective and ineffective trough Tacro concentrations was estimated to be at 6.8 μg/L (P < 10^-7). Compared with HCV- patients (n = 7), the 3 HCV+ patients had more variable associations between GcfDNA percentages and Tacro concentrations.

Conclusions: Direct measurement of graft integrity using GcfDNA was useful to confirm the lower limit of the therapeutic ranges for trough Tacro concentrations after LTx. It would probably be useful to do so also for other immunosuppressant drugs and after other solid organ transplants. The method might be especially useful to detect graft injury during immunosuppressant dose minimization strategies.

Key Words: graft-derived cell-free DNA, tacrolimus TDM, liver transplantation, graft injury

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INTRODUCTION

Drug blood concentrations have been used to individualize drug dosing because many drugs, including calcineurine inhibiting immunosuppressant drugs like tacrolimus (Tacro), exhibit large interindividual pharmacokinetic and pharmacodynamic (PD) differences.1–4 In solid organ transplantation, dosing of calcineurine inhibiting immunosuppressant drugs is adjusted on the basis of published therapeutic ranges (TRs). These TRs were established by consensus groups5 based on studies that statistically compared various outcomes (eg, rejection, graft survival, or toxicity) in groups of patients enrolled in randomized controlled trials6–7 with various measures of drug exposure (eg, trough Tacro concentrations). Dosing of liver transplantation (LTx) patients who were enrolled in these trials was adjusted based either on preselected Tacro concentration ranges or on surrogate biomarkers, such as liver enzymes, because until now no studies have demonstrated good correlation between PD markers and immunosuppressant efficacy,8 and there have been no practical biomarkers available that directly and specifically measure organ integrity. These Tacro TRs, although useful to establish general concentration targets that improve patient and graft survival,9 are imprecise, vary from center to center, and have limitations for managing individual patients.1,3,8,10

There are differences between various recommended trough Tacro TRs and the trough Tacro concentrations needed in individual LTx patients to prevent rejection while avoiding the complications of excessive immune suppression (eg, renal damage or infections).1,8,10 These individual differences are the result of many variables, including pharmacokinetic/PD differences in the recipients and in the Tacro assays used, the degree of donor/recipient match, the quality of the graft, other drugs and surgical procedures used, and the time after transplantation, graft injury

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LTx. A discussion of these differences is beyond the scope of this brief report.

In addition, previously, graft integrity has been measured only indirectly using conventional liver tests, such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, $\gamma$-glutamyltransferase, and bilirubin, which are not diagnostic of acute cellular rejection. Although complicated and expensive DNA sequencing methods were recently reported to be able to assess graft integrity, these methods were both very expensive and too time consuming to be clinically useful for LTx patient monitoring. However, recently, it has become possible at a reasonable cost to rapidly (within 1 day) and directly assess graft integrity by specifically measuring the percentage of plasma circulating GcfDNA in relation to that from the recipient. This technology, although not yet commercially available, has been shown to identify both the early rejection and the vascular causes of graft damage. This technique is being investigated in a large prospective multicenter study designed to identify ways to individualize treatment after solid organ (heart, kidney, and liver) transplantation. As part of these studies, we evaluated whether organ integrity measured by GcfDNA could be used to reexamine minimally effective trough Tacrolimus concentrations in adult LTx patients, which is the basis of the data presented here.

### MATERIALS AND METHODS

The studies described were approved by the local ethics committees. Briefly, adult organ transplant patients who gave their informed consent before LTx had blood samples collected at intervals after transplantation.

Data presented here are from 10 of the first 14 adult LTx patients who gave their consent to be studied. Data are not included from 3 hepatitis C virus (HCV) negative patients (1 woman aged 22 years and 2 men aged 47 and 57 years) who died before any samples were obtained and from a fourth subject (man, aged 62 years, retransplanted, hepatitis B positive) who was excluded on the basis of multiple factors, including but not limited to the collection of nontrough Tacrolimus concentrations, the use of extracorporeal support for extremely high (>40 mg/dL) bilirubin levels, use of unusually large amounts of biologic products (albumin, clotting factors, and transfusions) likely to contain extraneous DNA, and failure to be treated with the same drug regimen as the other 13 subjects. All the remaining 10 patients received immunosuppressant drugs as per the protocol used in Goettingen (available on request) that includes basiliximab, methylprednisolone, and mycophenolate mofetil in addition to Tacrolimus. The 10 subjects were all white and included 3 HCV+ patients and 8 men with a mean ± SD age of 56 ± 9.4 years (Table 1).

A total of 103 paired Tacrolimus and GcfDNA measurements from samples collected between days 5 and 30 after LTx were analyzed. Special DNA collection tubes were used as previously described.

Trough Tacrolimus concentrations were measured using a published LC-MS/MS method using a Quattro Premier XE Mass Spectrometer (Waters) by the University Medical Center Goettingen laboratory that is a regular participant in an external immunosuppressant drug quality control program.

GcfDNA was measured by the laboratory of Chronix Biomedical, Goettingen, Germany, using the proprietary, published droplet digital polymerase chain reaction (ddPCR) assay that uses selected single nucleotide polymorphisms (SNPs) to differentiate between donor and graft DNA isolated from plasma samples. Each subject had individualized ddPCR tests used to monitor GcfDNA percentages over time. In brief, for each patient, those SNP assays that allowed differentiation were selected using conventional real-time allele detection from a total of 41 SNP assays. Using those SNPs, the percentage of graft cfDNA was subsequently quantified by means of ddPCR in a QX100 system (Bio-Rad), as previously described. The imprecision of the GcfDNA assay was <15% even with low (2%) GcfDNA percentages.

A GcfDNA of 10% or less was set as indicating organ integrity because this is the percentage that was associated with good graft function in a different group of long-term, stable LTx subjects (n = 10) with no signs of rejection described elsewhere.

The number of samples with GcfDNA above and below 10% were categorized for patients whose simultaneous trough Tacrolimus concentrations were below versus those that were above the lower limit of the TR (8 $\mu$g/L) used by the transplant surgeons at our institution to adjust Tacrolimus dosing during the first 6 weeks after LTx. The statistical difference of the distribution into the 4 emerging categories was determined using the Fisher exact test. Comparisons were also made using the Youden index [\(\theta = \text{Tacro} \geq \text{delimit}(\text{GcfDNA} < 10) + \theta(\text{Tacro} \geq \text{delimit}(\text{GcfDNA} \geq 10))\)] and the $-\log$ Fisher $P$ values to define the best lower limit of the Tacrolimus TR at 0.1 $\mu$g/L intervals using a receiver operator characteristic curve method. The area under the curve of the receiver operator characteristic curve was calculated by the trapezoidal rule with 95% confidence interval (CI). To eliminate artificial peaks because of ties, the Youden and Fisher values were subjected to a Parzen–Rosenblatt smoothing ($\sigma^2 = 0.5$, $i = 3$).

### TABLE 1. Demographic and Clinical Information of the Patients Included in This Report

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>Weight (kg)</th>
<th>Diagnosis</th>
<th>Hepatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTx1</td>
<td>M</td>
<td>49</td>
<td>80</td>
<td>Cirrhosis</td>
<td>C</td>
</tr>
<tr>
<td>LTx2</td>
<td>M</td>
<td>41</td>
<td>80</td>
<td>Re-Tx</td>
<td>(sChol)</td>
</tr>
<tr>
<td>LTx3</td>
<td>M</td>
<td>68</td>
<td>84</td>
<td>Cirrhosis + HCC</td>
<td>–</td>
</tr>
<tr>
<td>LTx4</td>
<td>F</td>
<td>52</td>
<td>69</td>
<td>PCLD</td>
<td>–</td>
</tr>
<tr>
<td>LTx6</td>
<td>F</td>
<td>64</td>
<td>88</td>
<td>Cirrhosis + HCC</td>
<td>–</td>
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<tr>
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<td>M</td>
<td>44</td>
<td>65</td>
<td>Cirrhosis</td>
<td>C</td>
</tr>
<tr>
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<td>M</td>
<td>60</td>
<td>70</td>
<td>Cirrhosis</td>
<td>–</td>
</tr>
<tr>
<td>LTx10</td>
<td>M</td>
<td>64</td>
<td>82</td>
<td>Cirrhosis</td>
<td>C</td>
</tr>
<tr>
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<td>M</td>
<td>64</td>
<td>105</td>
<td>Cirrhosis + HCC</td>
<td>–</td>
</tr>
<tr>
<td>LTx14</td>
<td>M</td>
<td>58</td>
<td>85</td>
<td>Cirrhosis</td>
<td>–</td>
</tr>
</tbody>
</table>

C, HCV+; PCLD, polycystic liver disease; Re-Tx, repeat transplant; sChol, sclerosing cholangitis; HCC, hepatocellular carcinoma.
RESULTS

Although individual subjects had some differences in their minimum effective Tacrolimus concentration, for the entire group, there was a highly significant segregation (Fisher $P = 0.00002$) in these 103 Tacrolimus/GcfDNA pairs between trough Tacrolimus concentrations that were below the target concentration used in our institution during the first 6 weeks after LTx ($8–12 \text{ mg/L}$) and GcfDNA below 10% (Fig. 1). All but 2 samples from 7 HCV− patients had <10% GcfDNA when trough Tacrolimus concentrations were within the range of $8–12 \text{ mg/L}$, and these 2 exceptions were associated with only minor (ie, <15%) elevations of GcfDNA. The 3 HCV+ subjects had more variable results. The Tacrolimus dosages aiming for trough Tacrolimus concentrations in the TR were highly variable (Fig. 2), supporting the necessity for therapeutic drug monitoring.

The area under the curve of the receiver operator characteristic curve yielded its maximum (0.83; 95% CI, 0.67–0.93, $P<10^{-5}$) at a segregating value of 10% GcfDNA (Fig. 3). The 10% limit for GcfDNA had been chosen based on the data from a separate group of chronically stable adult LTx patients without clinical evidence of any complications during their post-LTx follow-up. Simulations with the dataset given here proved that this tentative limit gives the best separation for the evaluation of effective Tacrolimus concentrations. This supports the decision to use 10% GcfDNA as the limit to define LTx graft integrity.

The maximally effective minimum concentration in this group of subjects ($n = 10$) seemed to be 6.8 $\text{ mg/L}$ as defined by a maximized Youden index of 0.51 and the peak $-\log$ Fisher $P$ of 6.9 (Fig. 3). The odds ratio at this Tacrolimus concentration boundary was 10.7 (CI$_{95}$, 4.2–27.1). This translates for the patients in this dataset into a 4-fold (CI$_{95}$, 2.2–7.2) relative risk of having graft damage (ie, GcfDNA > 10%) with a Tacrolimus trough below 6.8 $\text{ mg/L}$. To assess the bias because of individual patients, a 100 × resampling of patient data was performed.

**FIGURE 1.** Comparison of predose Tacrolimus concentrations in micrograms per liter versus the percentages of cell-free DNA coming from the donor liver (GcfDNA) in adult patients ($n = 10$) during the first 5–30 days after LTx.

**FIGURE 2.** Boxplots of daily dosages of Tacrolimus between day 5 and 30 per individual patient. Median, interquartile range (boxes), and 5th to 95th percentiles (whiskers) are shown.
where \( \approx 50\% \) of patients were randomized into the dataset (5 or 6 per sampling). In each round, the best delimiter of Tacro concentration was calculated as above and recorded. The error was estimated with an SD of 0.4 \( \mu \text{g/L} \) (average: 6.9 \( \mu \text{g/L} \)), which suggests an influence of patients \( <10\% \), for this consideration.

**DISCUSSION**

Circulating cell-free DNA is being used in a number of clinical settings to evaluate organ function and tumor growth, \(^{11,17-19}\) but most methods used previously have required either gender mismatch, expensive and time-consuming massive sequencing, or chip-based methods that require donor DNA to be available. The simplified GcfDNA method used here promises to be a practical, rapid, and relatively inexpensive way to directly interrogate the integrity of transplanted organs, such as livers. \(^{12}\)

Although the method cannot be used to examine the upper limit of the TR, these results do support the reports\(^ {19,10}\) that some currently used target trough Tacro concentrations are above what is required to maintain graft function in LTx patients. Our results using this novel method to interrogate organ integrity are consistent with the most recent report by Rodríguez-Peralvarez et al\(^ {9}\) who used traditional graft survival assessment, showing that trough Tacro concentrations as low as 7 \( \mu \text{g/L} \) are safe and effective in LTx patients. These authors reported that liver graft survival was better with a mean trough Tacro concentration of 7–10 \( \mu \text{g/L} \) compared with \( <7 \mu \text{g/L} \) \((P = 0.008)\) and 10–15 \( \mu \text{g/L} \) \((P = 0.016)\). Tacro trough concentrations \( >7 \mu \text{g/L} \) on the day of protocol biopsy were associated with less moderate/severe rejection (23.8\%) compared with \( <7 \mu \text{g/L} \) (41.2\%, \( P = 0.004)\).\(^ {9}\) Using the GcfDNA as a measure of graft integrity, we calculated a limit of 6.8 \( \mu \text{g/L} \) for the lower trough Tacro concentration, well in line with this observation in a large study group.

Taken together, these data suggest that GcfDNA can be used to establish effective concentrations for LTx patients. In addition, the method seems to be a useful way to measure individual responses to immunosuppressive therapy. For example, previously we observed rapid decreases in GcfDNA after the introduction of methylprednisolone. \(^ {12}\) As such, it has great potential for “personalized immunosuppression.” Although studies with larger numbers and longer follow-up are needed, if these results are replicated and extended, GcfDNA could be used to select, adjust, minimize, or even discontinue certain immunosuppressant drugs in solid organ.
transplant patients. The timely and frequent application of this “liquid biopsy” could be especially useful to add some certainty about organ integrity during immunosuppressant drug minimization. This could help reduce both adverse drug events and rejection episodes by allowing clinicians to establish truly individualized minimal therapeutic targets rather than just unclear, broad population “ranges.”

CONCLUSIONS

Serial measurements of GcfDNA and trough Tacro concentrations were used in a small number (n = 10) of adult LTx recipients to confirm what minimally effective Tacro concentrations are during the first 5–30 days after LTx. The minimally effective trough Tacro concentrations in all subjects in this small population seemed to be 6.8 μg/L. Direct measurement of graft integrity using GcfDNA can be used to establish minimally effective trough Tacro concentrations and probably other immunosuppressants for individual patients after solid organ transplantation.

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REFERENCES