Monitoring of Donor-Derived Cell-Free DNA by Short Tandem Repeats: Concentration of Total Cell-Free DNA and Fragment Size for Acute Rejection Risk Assessment in Liver Transplantation

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Monitoring of graft function is essential during the first months after liver transplantation (LT), but current liver function tests (LFTs) lack the specificity and sensitivity to ensure an efficient diagnosis of acute rejection (AR). Recently, donor-derived cell-free DNA (ddcfDNA) has emerged as a noninvasive biomarker to assess graft integrity. This study evaluated the feasibility of measuring the ddcfDNA through short tandem repeat (STR) analysis by quantitative fluorescent-polymerase chain reaction (QF-PCR) and to assess the role of the concentration and fragment size of total cfDNA as AR biomarkers. The total concentration and fragment size of cfDNA and the ddcfDNA percentage were monitored in plasma of 20 patients without rejection and 7 patients with T-cell–mediated AR during the first 3 months after LT. The median ddcfDNA percentage was 3-fold higher before AR diagnosis (34.8%; $P < 0.001$) and moderately higher at AR confirmatory diagnosis (23.8%; $P = 0.049$) compared with that of nonrejector patients (10.6%), showing a better performance (area under the curve = 84.6%) than conventional LFTs to predict the risk of rejection within the first 2 weeks following LT. The fraction of 100-250-bp cfDNA fragments was higher at AR diagnosis compared with that of nonrejector patients (68.0% versus 57.9%, $P = 0.02$). STR amplification by QF-PCR may be an alternative strategy for rapid ddcfDNA quantification, which is easily implementable in clinical laboratories. The results of this pilot study indicate that ddcfDNA increases very early, even 1-2 weeks before the diagnosis of AR, and so it could be useful as a prognostic biomarker in improving patient risk stratification.

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Acute rejection (AR) remains a prevalent cause of morbidity and loss of graft functionality with an incidence of around 20% among liver transplantation (LT) patients.[1] Therefore, early detection is crucial to ensure the best outcome of therapeutic measures and to minimize the impact of AR. Current liver function tests (LFTs) used in clinical practice have a reduced sensitivity and specificity for an efficient AR diagnosis. Consequently, liver biopsy, an invasive procedure associated with potential complications, remains the gold standard for confirmatory diagnosis.

The cell-free DNA (cfDNA) has emerged as a potential noninvasive resource to assess the graft integrity and detect AR in LT among other solid organ

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; AR, acute rejection; BIL, bilirubin; cfDNA, cell-free DNA; CI, confidence interval; CsA, cyclosporine; DBD,

transplantation types.[2] The cfDNA is a double-stranded fragmented DNA present in biological fluids including plasma.[3] The total cfDNA concentration and specifically that fraction of cfDNA derived from the graft, named donor-derived cfDNA (ddcfDNA), are significantly increased in recipient's blood during allograft rejection.[4] In LT patients, a prospective observational multicenter study found that ddcfDNA quantification showed a better diagnostic performance to detect AR than conventional LFTs.[5] The ddcfDNA analysis detects rejection early and could provide a personalized approach for immunosuppressive therapy.[6]

Distinguishing the ddcfDNA from the cfDNA derived from recipient's cells is crucial for applying cfDNA-based strategies in the clinical management of transplantation patients. Several investigations have focused on the analysis of genetic differences between donor and recipient, such as sex determining region Y (*SRY*) gene,[7] human leukocyte antigen (HLA) genes,[8] or copy number deletion polymorphisms[9] using mostly quantitative polymerase chain reaction or Droplet Digital PCR (ddPCR). The study of Y-chromosome genes stands out for its easy interpretation, but it can be only applied in female recipients when the donor is a male. Currently, the genotyping of single-nucleotide polymorphisms (SNPs) by ddPCR[10,11] and sequencing by next-generation sequencing (NGS)[12,13] are the most promising strategies. However, the complexity of these protocols and the lack of ddPCR or NGS equipment in most conventional laboratories restrict their implementation in the clinical routine.

These limitations have encouraged us to investigate a novel strategy for ddcfDNA identification in plasma from the recipient based on the analysis of short tandem repeats (STRs), commonly known as "microsatellites." The STRs are highly polymorphic, and their analysis by quantitative fluorescent-PCR (QF-PCR) allows a rapid differentiation between genomes of distinct individuals.

The cfDNA fragment size has been associated with the origin and the release mechanism of cfDNA.[14] In healthy individuals, most cfDNA originates from the apoptosis of blood cells.[15] The standard cfDNA fragmentation pattern has a predominant peak around 166 bp and multiples thereof, corresponding to the typical DNA cleavage during apoptosis.[16] The cfDNA fragment size distribution has sprung up as a diagnostic and prognostic biomarker in patients with cancer,[17] but its role in LT patients has so far been poorly investigated.[18]

The aim of this study was to evaluate the feasibility of monitoring the ddcfDNA by STR analysis using a QF-PCR approach and to assess the role of the total cfDNA concentration and fragment size as prognostic or diagnostic biomarkers for AR in LT patients.

donation after brain death; DCD, donation after circulatory death; ddcfDNA, donor-derived cell-free DNA; ddPCR, Droplet Digital polymerase chain reaction; dsDNA, double-stranded DNA; GGT, gamma-glutamyltransferase; HLA, human leukocyte antigen; IQR, interquartile range; LFT, liver function test; LT, liver transplantation; MMF, mycophenolate mofetil; NGS, next-generation sequencing; NPV, negative predictive value; NS, not significant; PDN, prednisone; PPV, positive predictive value; QF-PCR, quantitative fluorescent-polymerase chain reaction; ROC, receiver operating characteristic; SD, standard deviation; SNP, single-nucleotide polymorphism; SRY, sex determining region Y; STR, short tandem repeat; TAC, tacrolimus; TCMAR, T-cell–mediated acute rejection.

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Esther Fernández-Galán, Joan Anton Puig-Butillé, and Mercè Brunet designed the study and planned the experiments; Mercè Brunet directed and supervised the project. Esther Fernández-Galán carried out the experiments, analyzed the data, and performed the statistics; Celia Badenas helped with technical details and interpretation of QF-PCR results; Esther Fernández-Galán wrote the original draft. Esther Fernández-Galán, Celia Badenas, Constantino Fondevila, Wladimiro Jiménez, Miquel Navasa, Joan Anton Puig-Butillé, and Mercè Brunet edited and reviewed the manuscript. All authors read and approved the final manuscript.

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Patients and Methods

STUDY DESIGN AND PATIENTS

This exploratory study included archived samples from 27 de novo adult LT patients corresponding to 20 patients without rejection (nonrejector patients) and 7 patients with AR (rejectors). Among rejectors, 4 were diagnosed with AR during the second week and 3 at the end of the first month after transplantation. Rejection was always confirmed by histological evaluation of graft biopsies (biopsy-proven AR). All patients presented T-cell–mediated AR (TCMAR), and the severity of the episodes was moderate according to the Banff classification.[19] The patient inclusion criteria for the study were the availability of at least 2 plasma samples during the posttransplantation period, including a sample at the time of AR diagnosis in rejectors. For all patients, a liver tissue sample from the donor was obtained for the quantification of ddcfDNA in the recipient's plasma.

The samples belong to a prospective observational study "Evaluation of alloreactivity and reversibility of fibrosis after treatment of hepatitis C in liver transplant patients" supported by a competitive grant from the Fondo de Investigaciones Sanitarias Instituto de Salud Carlos III (ISCIII, FIS PI14/01055, 2014), conducted in the Liver Transplant Unit and the Pharmacology and Toxicology Laboratory of Hospital Clínic de Barcelona (HCB/2014/0656). This study and the use of stored samples were approved by the Ethics Committee of the Hospital Clínic de Barcelona (HCB/2019/0522). Documented informed consent was obtained from each patient before participation in the prospective study.

COLLECTION OF PLASMA AND LIVER TISSUE SAMPLES

We included 59 plasma samples (40 from the nonrejector patients subgroup and 19 from the rejectors subgroup). A blood sampling protocol was carried out during postoperative visits from the first week to the third month after LT and when rejection was clinically suspected. In 71% (5/7) of AR patients, we collected a sample 1-2 weeks before rejection (pre-AR), a sample at confirmatory diagnosis (biopsy-proven AR), and a sample after antirejection treatment (post-AR; Fig. 1). In all patients with suspicion of AR, blood samples were collected on the day of the biopsy in the morning and prior to the biopsy; both (blood and tissue) samples were obtained within the 24 hours after clinical

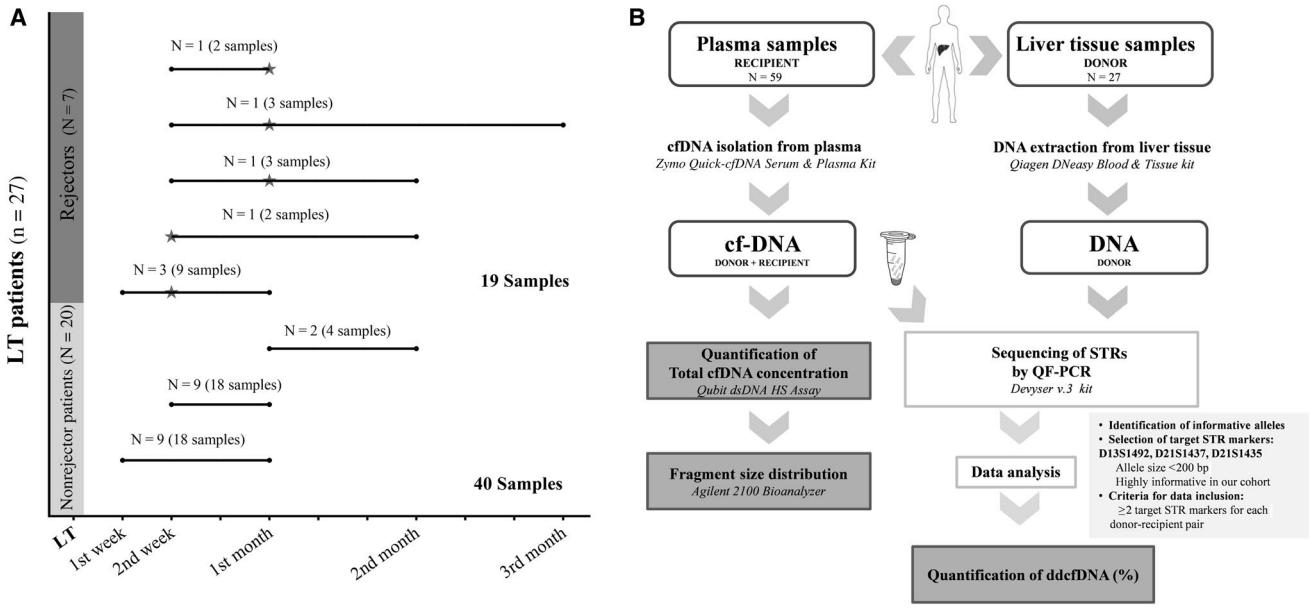


FIG. 1. Schematic representation of (A) the blood sampling in LT patients and (B) analytic workflow. (A) Blood collection points are represented as dots and samples collected at confirmatory AR diagnosis (biopsy-proven AR) are represented as stars. In nonrejector patients, 2 samples were collected at different time points during post-LT follow-up for each patient. For most patients with rejection (5/7), 3 samples were available: a sample previous to rejection, a sample at biopsy-proven AR, and a sample after antirejection treatment. (B) Analytic workflow for the quantification of total cfDNA, cfDNA fragment size, and ddcfDNA percentage in recipient's plasma.

suspicion of rejection. In all nonrejector patients, 2 blood samples at different time points during the same period after LT were available.

Preanalytical conditions of archived plasma samples, established in the prospective study, accomplished the recommendations for optimal cfDNA analysis.[20] Blood samples (3 mL) were collected into BD Vacutainer K_2EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and processed within 2 hours from collection. Plasma was obtained by centrifugation (3000*g*, 10 min), aliquoted, and stored at −80°C.

The donor DNA was extracted from tissue samples from liver grafts. All biopsy samples were obtained at the intraoperative evaluation of the donor organ and were immediately frozen at −80°C until analysis.

ISOLATION OF CFDNA FROM PLASMA AND DNA FROM LIVER TISSUE

The cfDNA was extracted from 1 to 2 mL of plasma using the Quick-cfDNA Serum & Plasma Kit (Zymo Research, Irvine, CA) following the manufacturer's recommendations. To avoid the contamination of genomic DNA by blood cells, we added an extra high-speed centrifugation step (16,000*g*, 10 min) before performing the manufacturer's protocol. Finally, cfDNA was eluted in 35 μL of elution buffer and stored at −80°C.

The donor DNA was extracted using the DNeasy Blood &Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The DNA concentration and purity (A_{260}/A_{280}) were measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). In addition, DNA concentrations were determined using the Qubit 3.0 Fluorometer and the double-stranded DNA (dsDNA) HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific).

CHARACTERIZATION OF CONCENTRATION AND FRAGMENT SIZE OF TOTAL CFDNA

The total cfDNA concentration in plasma was determined using the Qubit 3.0 Fluorometer and the dsDNA HS Assay Kit (Thermo Fisher Scientific). Results were reported as nanograms of cfDNA per milliliter of plasma, which were extrapolated from the concentration (C) measured in the eluted volume (EV) after cfDNA purification and the initial plasma volume (PV), using the following equation: ng/mL = C × EV/PV.

Fragmentation patterns were analyzed by electrophoresis with the Agilent 2100 Bioanalyzer on a High Sensitivity DNA chip (Agilent Technologies, Santa Clara, CA). The Agilent 2100 Expert software (version B.02.07) was used to obtain the fraction of cfDNA (%) and the average size (bp) for the ranks 100-250 bp and 250-700 bp.

SEMIQUANTIFICATION OF ddcfDNA IN PLASMA SAMPLES

STR Genotyping by QF-PCR

To quantify the ddcfDNA in the recipient's plasma, we genotyped 21 STR polymorphisms in the cfDNA and the donor DNA using the Devyser kit version 3 (Devyser Diagnostics, Hägersten, Sweden) by QF-PCR following manufacturer's instructions. This kit is designed for diagnosing prenatal aneuploidy in genomic DNA and analyses STRs located on chromosome 13 (D13S742, D13S634, D13S628, D13S305, D13S1492), chromosome 18 (D18S978, D18S535, D18S386, D18S976, GATA178F11), chromosome 21 (D21S1435, D21S11, D21S1411, D21S1444, D21S1442, D21S1437), chromosome X (DXS1187, XHPRT, DXS2390), chromosomes X/Y (DXYS267, DXYS218), and nonvariable markers for chromosomes X or Y or both (AMELXY, ZFXY, and SRY). Fragment analysis was performed by capillary electrophoresis separation using an ABI3130xL analyzer (Applied Biosystems, Waltham, MA).

STR Data Analysis

For data analysis, we used the GeneMapper version 4.0 software (Thermo Fisher Scientific). We determined the fragment size for each allele at each STR locus and integrated its peak area (proportional to the amount of DNA). We selected the informative alleles (ie, not shared between donor and recipient) necessary to quantify the percentage of ddcfDNA.

$$\text{ddcfDNA}(\%) = \frac{\text{Area under donor peak(s)}}{\text{Area under donor peak(s)} + \text{Area under recipient peak(s)}} \times 100$$

Informative STR markers contain at least 1 informative allele from the donor and 1 informative allele from the recipient. We discarded the STR containing artifacts or overlapping peaks that did not clearly differentiate between donor and recipient.

The results showed that STRs with alleles of length >250 bp did not amplify homogeneously due to the fragmentation of cfDNA. In addition, most of the markers located in sexual chromosomes were uninformative. Therefore, we selected shorter (<200 bp), sex-independent STRs with robust signals. The selected target STR were D13S1492 (size range = 119-155 bp), D21S1437 (115-145 bp), and D21S1435 (170-199 bp). At least 2 of these STRs must be informative for data analysis. By applying these criteria, we quantified the ddcfDNA in 100% (7/7) of AR patients and 75% (15/20) of nonrejector patients.

QUANTIFICATION OF ddcfDNA BY NGS

In 38 samples from 17 patients (10 nonrejector patients and 7 rejectors), we determined the percentage of ddcfDNA using the commercial kit AlloSeq cfDNA (CareDx, Inc., South San Francisco, CA) following the manufacture's protocol. The kit is a targeted NGS assay which employs highly polymorphic SNPs to quantify ddcfDNA. Libraries were sequenced using a Miseq instrument (Illumina, San Diego, CA). For all patients we sequenced the DNA from the tissue biopsy and the cfDNA from plasma. Data was analyzed by AlloSeq cfDNA software provided by the manufacturer.

STATISTICAL ANALYSIS

Comparisons between groups were performed using Student *t* test or the nonparametric Mann-Whitney U test depending on the data distribution. For categorical variables, we used the chi-square test. We assessed the correlation between variables using Spearman's or the Pearson coefficients, and by applying the logarithmic transformation to fit data to a normal distribution where it was appropriate. The set of "rejector" samples included both samples collected prior to rejection and samples collected at the rejection point, excluding samples collected after antirejection treatment. For data analysis, we used SPSS statistical software version 24.0 (IBM Corporation, Armonk, NY), R-studio software version 1.2.5033 (RStudio, Boston, MA), and for drawing graphs GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA), with *P* values of 0.05 or less denoting statistical significance. We collected clinical, demographical, and laboratory data (immunosuppressant drugs and LFT concentrations) retrospectively. To compare the prognostic or diagnostic abilities between studied and conventional biomarkers (LFTs), we used a receiver operating characteristic (ROC) curve analysis. Data were included in the comparative analysis when we had the results of all the biomarkers for that sample. The optimal cutoff value was determined by the Youden Index.

Results

CLINICAL AND DEMOGRAPHIC CHARACTERISTICS OF LT RECIPIENTS AND DONORS

First, we evaluated the differences in the clinical and demographic characteristics of LT patients (Table 1).

TABLE 1. Demographic and Clinical Characteristics of the 27 LT Patients and the Corresponding Donors

Characteristic	Total (n = 27)	Nonrejector patients (n = 20)	Rejectors (n = 7)	*P* Value
Recipient sex: male	22 (81)	16 (80)	6 (86)	0.74
Donor sex: male	13 (48)	9 (45)	4 (57)	0.58
Recipient age, years	55 (50-57)	55 (51-57)	50 (43-56)	0.28
Donor age, years	58 (53-63)	60 (56-66)	53 (50-61)	0.21
Type of donor				0.43
DBD	22 (81)	17 (85)	5 (71)	
DCD	5 (19)	3 (15)	2 (29)	
Cold ischemia time, minutes	500 (420-552)	505 (428-552)	430 (378-725)	0.79
Immunosuppressive regimen				0.36
TAC + PDN	7 (26)	6 (30)	1 (14)	
TAC + MMF + PDN	13 (48)	8 (40)	5 (71)	
CsA + MMF + PDN	7 (26)	6 (30)	1 (14)	

NOTE: Quantitative variables are presented as median (interquartile range); all other data are presented as n (%). Statistical differences between groups were assessed with the Mann-Whitney U test. For categorical variables, intragroup relative frequencies were compared among groups by chi-square test.

Overall, 81% (22/27) LT recipients were males with a median age of 55 years. None of the patients had human immunodeficiency virus or active hepatitis C virus infection. Regarding the immunosuppressive regimen, 74% (20/27) and 26% (7/27) of the patients received tacrolimus- or cyclosporine-based immunosuppression, respectively.

All donors were deceased (81% donation after brain death), and 48% (13/27) were males with a median age of 58 years. Rejector and nonrejector patients showed similar clinical and demographic characteristics.

TECHNICAL VERIFICATION OF METHODS USED FOR QUANTIFICATION OF TOTAL cfDNA AND SEMIQUANTIFICATION OF ddcfDNA

We verified the performance of the Qubit dsDNA HS assay for cfDNA quantification, and our results (limit of quantification = 1 ng/mL and intra-assay CV ≤ 5%) were similar to those reported by the manufacturer. The linear regression analysis showed slopes from 0.99 to 1.00 with intercepts from −0.01 to 0.40, indicating that the linearity was appropriated in the range of concentrations evaluated.

The evaluation of the STR analysis by QF-PCR was carried out in 6 samples per duplicate. While samples with ddcfDNA >5% showed a good reproducibility (CV ≤ 10%), samples with ddcfDNA <5% showed greater variability. In addition, we analyzed 38 samples (ddcfDNA range by QF-PCR analysis = 2.2%-46.7%) by using a commercial kit based on NGS. Overall, the fraction of ddcfDNA showed an excellent correlation between both methodologies (r = 0.94, P < 0.001, Supporting Fig. 3). However, there was a lack of correlation between data from QF-PCR and NGS in samples with ddcfDNA <5%, suggesting a limited capacity for QF-PCR to accurately quantify ddcfDNA below 5%. This was in accordance with the previously reported sensitivity for QF-PCR methods (≈5%).[21]

EVOLUTION OF TOTAL cfDNA CONCENTRATION AFTER TRANSPLANTATION

We evaluated the total cfDNA concentration in 93% (55/59) of samples. In 4 samples from 3 nonrejector patients and 1 rejector patient after antirejection treatment, the total cfDNA concentration was below the assay limit of detection.

In nonrejector patients, we observed high total cfDNA concentrations (median, 57.9 ng/mL) during the early postoperative period; in 82% (14/17) of patients it was over 20 ng/mL, followed by a decrease and reaching a plateau of about 20 ng/mL after 2 weeks (Fig. 2A). One month after LT, cfDNA median concentration decreased 3 times compared with that observed during the first week (P < 0.001), and the levels maintained stable 2 months after LT. In this group, total cfDNA concentration showed a moderate negative correlation with time (days) after LT (r = −0.40; P = 0.02; Supporting Fig. 1). Thus, the time since transplantation should be considered for evaluating the cfDNA concentration in LT patients.

In rejector patients, high total cfDNA concentration was observed during the first week (median, 78.8 ng/mL) after LT but in contrast to nonrejector patients, levels remained high 1 month after transplantation (median, 88.3 ng/mL).

We observed that the total cfDNA concentration was higher in rejector patients before antirejection treatment compared with that of nonrejector patients (median, 51.9 ng/mL versus 24.2 ng/mL; P = 0.02). Differences were caused by 3-fold higher levels 1-2 weeks before the AR confirmatory diagnosis compared with those in nonrejector patients (median, 69.1 ng/mL versus 24.2 ng/mL; P = 0.004), and by moderately higher levels at biopsy-proven AR time point (33.0 ng/mL versus 24.2 ng/mL). After antirejection treatment, rejector patients showed similar levels to nonrejector patients (16.0 ng/mL; Fig. 2B). However, these results should be interpreted with caution because when we restricted the analysis to the first 2 weeks after LT, we did not find significant differences between patients at pre-AR (median, 69.1 ng/mL; P = 0.09) or at biopsy-proven AR (median, 25.4 ng/mL; P = 0.32) and nonrejector patients (median, 35.6 ng/mL).

ASSOCIATION OF LT PATIENT STATUS WITH TOTAL cfDNA FRAGMENT SIZE

We explored whether the fragment size distribution of cfDNA was associated with AR. We evaluated the fragment size distribution in 97% (57/59) of samples. In 2 samples from 2 nonrejector patients, the fragment size was not accurately determined and was excluded

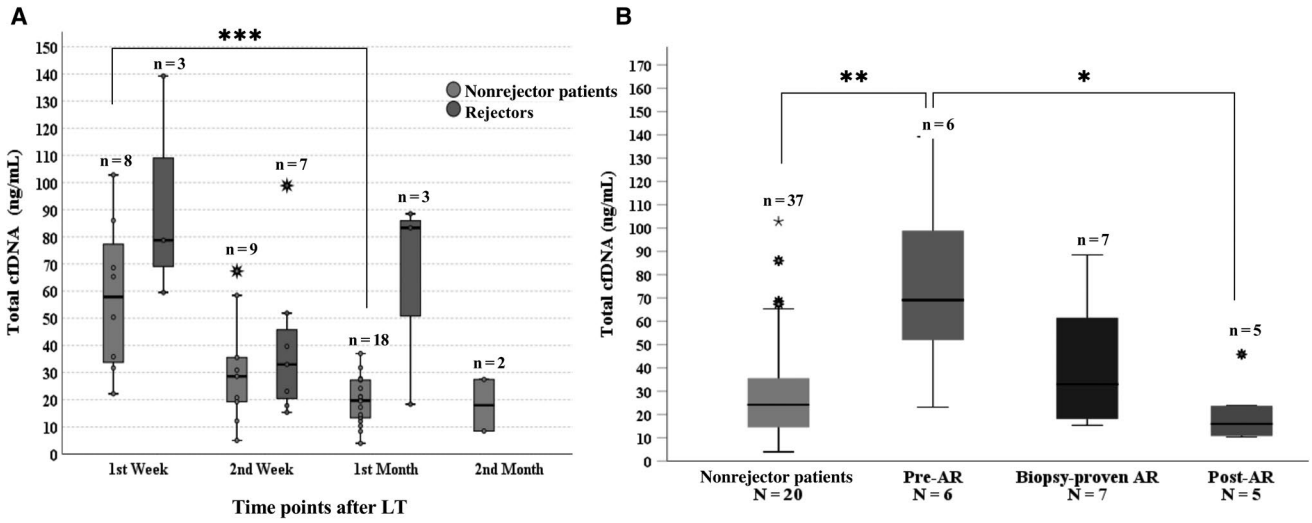


FIG. 2. (A) Evolution of total cfDNA in plasma from LT patients after transplantation and (B) differences by clinical status. Boxes represent median and IQR; whiskers extend to the largest value no further than ±1.5 × IQR; N indicates number of patients; and n indicates number of samples. * outliers, star in Fig. 2B represents an extreme value. (A) Total cDNA concentration in plasma from 20 nonrejector patients and 7 rejectors. Each "rejector" boxplot includes samples from patients who had a biopsy-proven AR diagnosis at this time point plus samples from patients who exhibited rejection in a later profile, excluding samples after antirejection treatment. (B) We observed a higher median cfDNA concentration in rejector patients before the AR diagnosis (Pre-AR) compared with that in nonrejector patients (69.1 ng/mL versus 24.2 ng/mL; $P = 0.004$). After antirejection treatment (post-AR) the cfDNA decreased to levels comparable to nonrejector patients. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

for the analysis. In nonrejector patients, size distribution was similar over time (range 100-250 bp = 57.0-63.0%; range 250-700 bp = 18.0%-22.0%).

Interestingly, rejector patients showed a higher fraction of 100-250-bp fragment size at the moment of AR confirmatory diagnosis compared with nonrejector patients (68.0% versus 57.0%; $P = 0.02$; Table 2). After resolution of rejection, the percentage of shorter fragments decreased to values similar to those of nonrejector patients.

ANALYSIS OF DDCFDNA AS A PROGNOSTIC AR BIOMARKER

The percentage of ddcfDNA was quantified in LT recipient's plasma genotyping of STR by QF-PCR in 100% (19/19) of samples from rejector patients and 75% (30/40) of samples from nonrejector patients (Supporting Tables S1 and S2).

In nonrejector patients, the ddcfDNA levels decreased during the follow-up ($r = -0.37$; $P = 0.01$), (Supporting Fig. 2). Mostly, patients showed 20.0% or less ddcfDNA during the early postoperative period except in 5 patients including 4 who presented high values: 50.0% (day 8), 32.5% (day 7), and 45.2% (day 16). These patients presented altered LFTs at that moment, and the ddcfDNA levels decreased during the follow-up to 20.3%, 18.3%, and 2.4%, respectively. At the end of the first month, the ddcfDNA percentage was below 10% in 73% (11/15) of patients and ddcfDNA was not detected in 3 patients. Data obtained at the second month of follow-up were excluded due to noncompliance with 1 eligibility criterion (≥2 target STR markers).

By contrast, we observed similar ddcfDNA levels after transplantation until the end of the first month in rejector patients. The median percentage of ddcfDNA was 26.5% in the first week, 32.8% in the second week, and 23.8% at the end of the first month, indicating that the ddcfDNA percentage did not decrease during the first month after transplantation.

The ddcfDNA percentage showed a moderate positive correlation with total cfDNA ($P = 0.01$) when we compared data from all samples (n = 42). However, we did not find correlation if we analyzed data from samples collected during the 2 first weeks after transplantation (n = 24), suggesting that they follow a different pattern during this period.

We assessed the role of ddcfDNA percentage as a prognostic or diagnostic biomarker of AR. Median

TABLE 2. Fragment Size Distribution of cfDNA in LT Patients by Clinical Status

	cfDNA 100-250 bp			cfDNA 250-700 bp			
	Median Percentage (IQR)	*P* Value	Mean Size ± SD	*P* Value	Median Percentage (IQR)	*P*-Value	Mean Size ± SD	*P*-Value
Differences by patient status								
Nonrejector patients (number of patients = 20, number of samples =38)	57.0 (52.0-67.0)		176.9 ± 3.0		22.0 (20.0-24.0)		431.8 ± 14.1	
Rejectors (number of patients = 7, number of samples = 19)								
Pre-AR (number of samples = 6)	65.0 (59.0-80.0)	0.17	175.2 ± 2.5	0.20	22.5 (12.0-24.0)	0.83	420.7 ± 20.1	0.24
Biopsy-proven AR (number of samples = 7)	68.0 (65.0-78.0)	0.02	176.0 ± 2.9	0.54	20.0 (14.0-24.0)	0.29	423.9 ± 11.1	0.13
After AR (number of samples = 6)	51.0 (43.0-80.0)	0.58	177.7 ± 5.7	0.72	25.0 (12.0-28.0)	0.56	419.2 ± 40.0	0.14

NOTE: Differences were assessed using the Mann-Whitney U test for median percentage and Student *t* test for mean size.

ddcfDNA was higher in rejector patients before the antirejection treatment compared with that of nonrejector patients (31.5% versus 10.6%; $P < 0.001$), reaching the highest levels (3-fold) 1-2 weeks prior to AR diagnosis (34.8% versus 10.6%; $P < 0.001$; Fig. 3A). The ddcfDNA percentage at "biopsy-proven AR" was also higher compared with nonrejector patients (23.8% versus 10.6%; $P = 0.049$). However, 3 rejector patients presented a ddcfDNA percentage ≤15% at this point. After antirejection treatment, the ddcfDNA levels decreased in all patients and reached levels similar to those observed in nonrejector patients 1 month after LT (6.7% versus 5.5%).

Next, we analyzed the ddcfDNA variability by patient status based on the time since LT (Fig. 3B). In the early postoperative period, the ddcfDNA showed greater differences than the total cfDNA concentration between rejectors and nonrejector patients. The median ddcfDNA during the first 2 weeks after LT was significantly higher ($P = 0.02$) in rejector patients at pre-AR profile (median, 34.8%) compared with that of nonrejector patients at the same period (14.5%). One month after transplantation, we also found higher ddcfDNA levels at biopsy-proven AR profile (median, 23.8% versus 5.5%; $P = 0.04$).

COMPARISON OF TOTAL cfDNA AND ddcfDNA WITH CONVENTIONAL BIOMARKERS

We found a strong positive correlation between aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT) and ddcfDNA; a moderate positive correlation with total cfDNA; and no correlation with the fraction of 100-250-bp cfDNA fragments (Fig. 4A).

We compared the ddcfDNA and total cfDNA capacity to identify patients at risk of rejection (prognostic) or patients with active rejection (diagnostic) with that of LFTs using the ROC analysis (Fig. 4B).

Including data from patients at biopsy-proven AR point (n = 7) and data from patients free of rejection collected at the same period (n = 27), the ddcfDNA showed an acceptable diagnostic performance, similar to that shown by LFTs. The area under the curve (AUC) was 77.2% (95% CI, 58.7%-95.8%). Using an optimal cutoff value of 13.8% the sensitivity and the specificity were 85.7% and 63.3%; the positive predictive value (PPV) and the negative predictive value (NPV) were 35.3% and 95.5%, respectively.

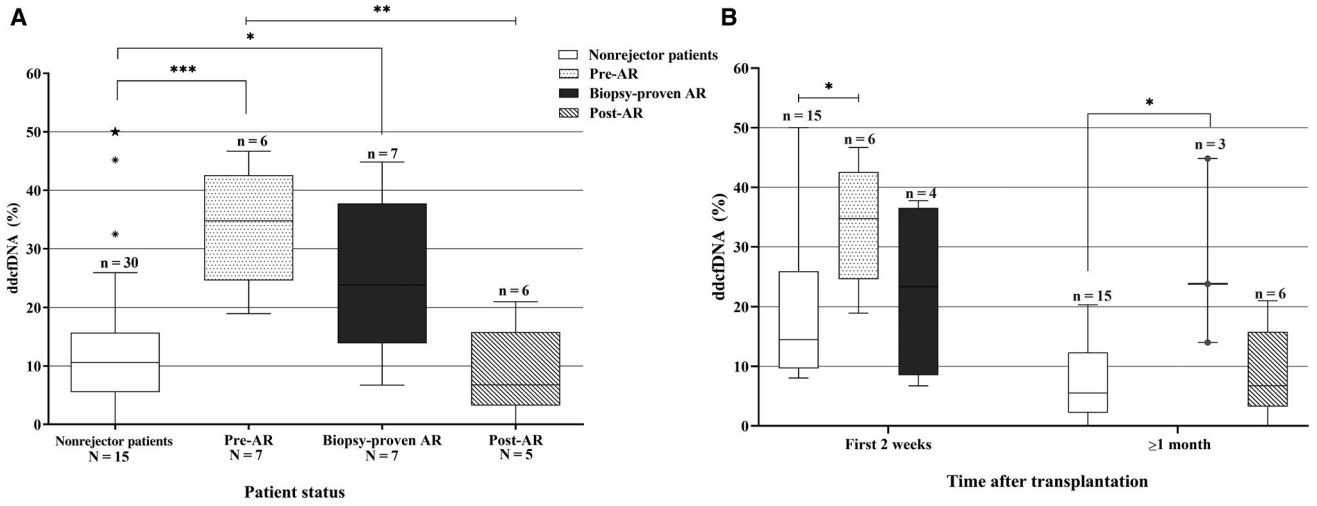
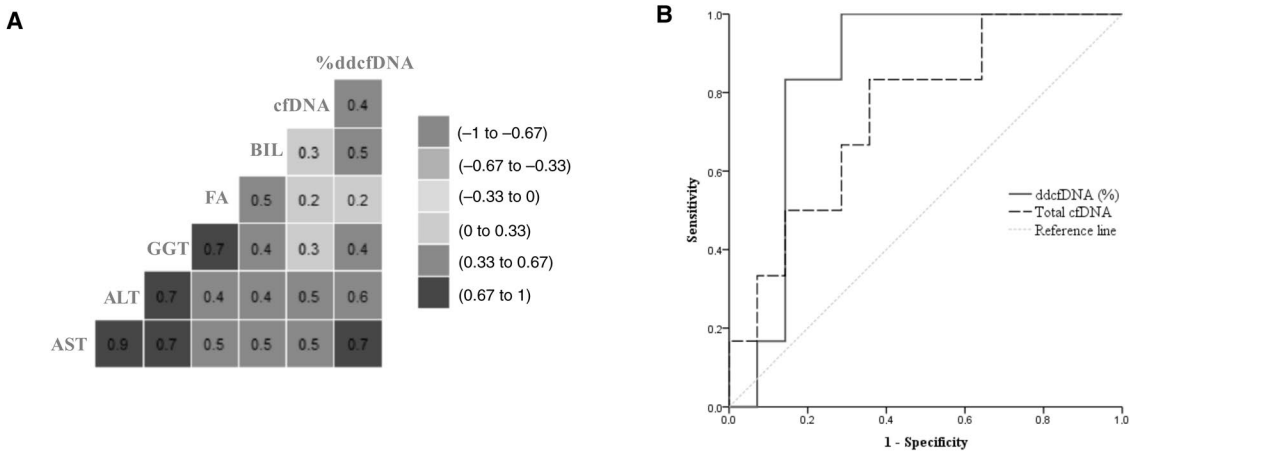


FIG. 3. Differences in ddcfDNA levels in plasma from LT patients by (A) clinical status and (B) time after LT. Boxes represent median and IQR; whiskers extend to the largest value no further than ±1.5 × IQR; N indicates number of patients; and n indicates number of samples. * outilers, star represents an extreme value. (A) Higher median ddcfDNA (%) was observed in rejectors before the diagnosis of AR (Pre-AR) compared with that in nonrejector patients. At the biopsy-proven AR point, it was also significantly higher than that in nonrejector patients and after antirejection treatment (post-AR) the ddcfDNA decreased to levels comparable to the nonrejector patients group. (B) Comparative analyses were carried out according to the time after LT. Data included in the "Pre-AR" or "biopsy-proven" boxplots were compared with data from nonrejector patient's samples during the same period. Statistically significant differences were found at pre-AR and at biopsy-proven AR profiles compared with each respective control group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.



Total cfDNA	AST	ALT	GGT	ALP	BIL
R (Spearman's)	0.47	0.48	0.31	0.19	0.25
P value	<0.001	<0.001	0.03	0.16	0.06
% ddcfDNA	AST	ALT	GGT	ALP	BIL
R (Spearman's)	0.67	0.63	0.39	0.20	0.45
P value	<0.001	<0.001	0.007	0.17	0.001

Variables	AUC (%)	95% CI	*P* value
% ddcfDNA	84.6	66.1-100.0	0.02
Total cfDNA	76.9	54.7-99.2	0.07
AST	67.9	37.5-98.4	0.22
ALT	53.8	20.2-87.4	0.79
GGT	48.7	19.0-78.5	0.93
ALP	53.8	28.0-79.7	0.80

FIG. 4. Comparison of total cfDNA and ddcfDNA with conventional biomarkers. (A) Correlation matrix between studied biomarkers and conventional LFTs. (B) ROC curve for the prognostic capacity. For this analysis, we included data from rejector patients prior to rejection (n = 6) and data from nonrejector patients (n = 13) at the same period (the first 2 weeks after LT).

For prognostic analysis, we included data from rejector patients collected 1-2 weeks prior to rejection (n = 6) and data from nonrejector patients at the same period (n = 13). The ROC analysis showed that ddcfDNA had a better performance than LFTs; AUC of 84.6% (95% CI, 66.1%-100%). Using an optimal cutoff value of 16.9%; the sensitivity and specificity were 100% and 67.7%, respectively; and the PPV and the NPV were 55.5% and 100%, respectively.

Discussion

In this work, we successfully detected ddcfDNA using STR amplification by QF-PCR in plasma from adult LT recipients. Our results in terms of clinical significance of ddcfDNA dynamics in LT are in agreement with a previous study carried out by ddPCR, which has demonstrated its usefulness in a prospective multicenter study.[5] Further, we found a high correlation between the percentage of ddcfDNA obtained by QF-PCR and that by a complex molecular strategy such as NGS. Altogether, these data support the hypothesis that QF-PCR could be an alternative method for the rapid quantification of ddcfDNA in LT recipients, especially in laboratories without NGS or ddPCR equipment and trained personnel for interpretation of its results. The QF-PCR is a robust, low-cost method with a short turnaround time (<3 hours) and is suitable for automation and easily implementable in clinical laboratories. However, it provides semiquantitative results and has a lower sensitivity compared with ddPCR or NGS. The sensitivity of QF-PCR is not a major limitation in LT recipients, which usually shows elevated ddcfDNA levels during AR, but this limits its application to other types of solid organ transplantation with lower expected levels of ddcfDNA, such as kidney,[22] heart,[23] or lung transplantation.[24]

In our study, the total cfDNA and ddcfDNA levels were significantly higher in rejector patients compared with nonrejector patients, and the maximum levels of both biomarkers occurred 1-2 weeks before the AR episode was confirmed. This is in accordance with Schütz et al.[5] who found that ddcfDNA increases even 8-15 days prior to confirmatory diagnosis by biopsy. We found moderately higher ddcfDNA levels at biopsy-proven AR due to low levels in 3/7 patients at this time point. Although ddcfDNA <10% has been reported previously in some patients with confirmed AR,[5] this fact is more prevalent in our cohort. It is important to highlight that although ddcfDNA levels were not so high at AR diagnosis profile, we also found significant differences when we compared the median percentage of ddcfDNA with that of nonrejector group. This trend contrasts with other biomarkers in LT which reach the maximum peak at the time of AR diagnosis.[25] For this reason, it is necessary to carry out further studies to evaluate the interplay between recipient immune response and graft in order to understand the biomarker kinetics during the rejection episodes. Eventually, we observed that ddcfDNA decreased promptly after antirejection treatment, which reinforces the evidence that high-dose steroid bolus for the AR treatment causes a rapid decrease in ddcfDNA levels.[26]

We detected high levels of total cfDNA and ddcfDNA in nonrejector patients during the first 2 weeks after transplantation, followed by a progressive decrease until the first month. This time dependence was confirmed by a significant negative correlation between concentrations of total cfDNA, ddcfDNA, and time after transplantation. Increases in cfDNA[27] and ddcfDNA[28] have been associated with systemic inflammation and the ischemia-reperfusion injury—common during the early postoperative period; therefore, these are important clinical confounding factors that can affect the performance of both biomarkers to detect AR during first 2 weeks after LT.

Consequently, the time since transplantation, as in other biomarkers, is a relevant covariate that should be considered when evaluating cfDNA and ddcfDNA. In this study, all patients rejected the transplant before the end of the first month; thus, the monitoring of biomarkers prior to rejection or at the rejection point also comprises the early postoperative period, which could reduce the obtained specificity for these biomarkers. Even in this period, the ddcfDNA level was significantly higher in patients who develop AR compared with that of nonrejector patients. The ddcfDNA presented better performance than LFTs and cfDNA to classify patients at risk of rejection within the first 2 weeks after LT. Furthermore, the ddcfDNA seems more prognostic (AUC = 84.6%) than diagnostic (AUC = 77.2%). The main advantage of ddcfDNA compared with tissue biopsy is that it is a noninvasive procedure that can be repeated easily, with a shorter turnaround time. These advantages together with its high NPV (100%) indicate that ddcfDNA monitoring can be very useful to rule out patients with very low risk of rejection, avoiding unnecessary diagnostic procedures and biopsies.

The cfDNA fragment size differs under pathological conditions,[29] and it offers complementary information regarding the molecular processes involved in its secretion. In a recent publication, a high proportion of shorter fragments (105-145 bp) have been associated with graft injury in living-related LT.[18] However, limited information exists about the cfDNA fragment size in LT, and further studies evaluating the clinical significance of distinct range of cfDNA fragment size are needed. Our study evaluated for the first time the cfDNA size in a cohort including LT patients with rejection and showed a higher proportion of shorter fragments during AR, which suggests that cfDNA fragments derived from the graft are shorter than those derived from the recipient.

The main limitations of our study are the sample size and the single-center retrospective design. All patients presented moderate TCMAR within the first month after transplantation. This is the most frequent form of liver rejection, but it is important to point out that our findings cannot be extrapolated to other grades or types of rejection, such as subclinical or chronic rejection. As our method is a relative quantification, we should considerer that ddcfDNA fraction can also be affected by the variability in recipient cfDNA concentration.[30]

Further studies with larger sample size are required to validate our results and to explore the clinical relevance of monitoring cfDNA concentration, fragment size, and ddcfDNA in LT patients.

Conclusion

This pilot study indicates that ddcfDNA increases early, even before the confirmatory diagnosis of AR, suggesting its utility as a prognostic biomarker of rejection risk in LT patients. The proposed methodology, based on STR analysis by QF-PCR, might be a cost-effective alternative for rapid ddcfDNA quantification in clinical laboratories. This approach may be an excellent support for clinical decision making, facilitating timely therapeutic interventions in LT patients.

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