Absolute Quantification of Graft derived cell-free DNA (GcfDNA) early after Liver Transplantation (LTx) using droplet Digital PCR

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Abstract

Background: The diagnostic value of GcfDNA as measure of graft integrity after 1h has been recently proven [1,2]. The yin and yang of using percentage values vs. absolute GcfDNA quantification is, nevertheless, under discussion [3]. Where the ratio of graft to host cfDNA has analytic advantages by eliminating disturbing variables, such as DNA extraction efficiency, variabilities in host cfDNA may obfuscate the view on the engrafted organ. The early phase after LTx was used as model to interrogate whether the percentage or absolute plasma concentration of GcfDNA is a more valuable graft integrity measure.

Methods: GcfDNA was quantified by droplet dPCR (BioRad) as described [1]. A synthetic sequence of non-human origin (average length of cfDNA) was spiked into human plasma, and quantified with ddPCR after DNA extraction in one fluorescence channel. The total cfDNA was quantified using two human genomic ddPCRs in the second channel as copies/mL (cp/mL). Total cfDNA was calculated using the spiked in copy number divided by 100. A Bland-Altman analysis was performed to assess the extraction efficiency in each batch. GcfDNA concentration was defined as the total cfDNA/cp/mL x GcfDNA%. Plasma samples from five patients were monitored during the first 10 days after LTx. GcfDNA was measured via different extraction methods to correct for extraction efficiency [4].

Results: Ten repeated extractions of the same plasma pool from healthy volunteers yielded an average of 1069 diploid genomic cp/mL plasma with a CV of 7.5%. Of 185 samples six showed a low (<50%) extraction efficiency; the remaining had an average of 67%±9%. The total cfDNA was highly variable peaking at day 3 but decreasing to 2.9±0.5 cp/mL at day 10. The respective GcfDNA was 3.1±1.9±0.8 cp/mL (6h) and 1.5±1.6±0.9±0.1 cp/mL (day 10). The correlation between GcfDNA% and GcfDNA(cp/mL) values was weak (r=0.61,p<0.05). A comparison of the AUC (±1-5) of AST with GcfDNA percentage and concentration showed a better association with absolute GcfDNA (r=0.65,p<0.05) compared to percentages (r=0.31,p=0.27). The minimal initial half was 1.3±0.6 ±GcfDNA(cp/mL) and 2.9±1.6±1.6 GcfDNA(cp/mL), compared to 2/day±1.2 for AST.

Conclusions: A robust and precise ddPCR method for absolute quantification of GcfDNA was developed, combining the analytical advantages of graft/host ratio (e. g. eliminating possible bias from interferences), with a robust quantification of total cfDNA. The GcfDNA concentration seems better associated with AST-values early after LTx and showed a more rapid dynamics than GfCDNA. Even though the initial post LTx phase, with highly variable amounts of total cfDNA, is particularly complicated, this method may also provide a better view on graft integrity in other situations, where absolute quantification is required.

Introduction

It has been shown in several studies that the quantification of graft derived circulating free DNA (GcfDNA) has the clinical potential for interrogating the integrity of the transplanted organ using this “fingerprint approach” ([3]. We have shown that GcfDNA is useful to follow the minimal required dosage of immunosuppressive drugs in the early post transplant phase [2]. However, the use of percentages can be misleading of non-transplant related circumstances lead to an increase of host cfDNA, over the amount that is usually observed in complication-free solid organ recipients. Furthermore, the immediate post engraftment phase is complicated by the recovery from immunosuppression-refractory injury, which can lead to extreme cfDNA concentrations. The minute amounts of cfDNA that can be extracted from plasma require careful control of extraction efficiency and analytical methods that yield precise measures with minimum sample input. The aim of this work was to establish a robust system for reliable quantification of GcfDNA expressed as absolute concentration [cp/mL], controlling the pre-analytical error and the comparison of plasma samples from the engrafted GcfDNA non-intact disease.

Materials and Methods

Patients, Materials and Methods

Blood samples from patients after LTx (LTx), heart (HTx) and kidney (KTx) were drawn according to IRB approved protocols. 288 samples from 33 LTx were included. For the cfDNA extraction investigations normal volunteers are used. EDTA-whole blood was drawn and processed within 4 hours and stored frozen at -80°C until extraction. For LTx patients cfDNA tubes (Ismi) Streck Inc. were used for a subset of draws.

cfDNA Extractions

Before extraction all plasma samples were centrifuged at 4000 x g for 20 min at 4°C. Two cfDNA extraction methods were applied: QiAamp Circulating Nuclear Acid Extraction kit (QIAGEN), Roche’s High Pure Viral Extraction Kit (HEP). The kit was used with 1/3 and without the recommended carrier RNA. Extractions were performed on the same plasma pool on three different days. Two different volumes were extracted from each kit: 2.5 mL and 5 mL for QIAGEN, 1.5 mL and 2.5 mL for Roche. ddPCR were performed in triplicate for each extraction day. For the in-essay assessment of the extraction yield, an artificial spike added to the plasma immediately before adding the protease-binding buffer. The spike consists of a non-human derived 320bp DNA that is prepared by PCR on the vector-cloned fragment. The product was stored in 1.4X-Lyophil solution and was diluted freshly prior to extraction.

dPCR Assays

All primers were purchased from Sigma-Aldrich and probes from MWG-Biotech Euromed. ddPCRs were performed using a Bio-Rad QX100/QX200 (Bio-Rad) and data was analyzed using the QuantaSoft version 1.38 software (Bio-Rad) and exported to Excel for further calculations. ddPCRs were in 1x ddPCR Supermix for Probes or EuGene mastermix as appropriate (Bio-Rad).

Absolute cfDNA quantification

For the quantification of absolute GcfDNA two assays each targeting one single copy genomic locus were combined in one ddPCR (HEX channel) together with one assay targeting the artificial spike product (FAM channel). GcfDNA [%] was measured as described elsewhere ([1]). The GcfDNA (cp/mL) was calculated by multiplying the GcfDNA [%] with the cfDNA (cp/mL) (Figure 1). 20 µl of the spike-dilution used for the respective extraction were diluted to a final volume of 50 µl. Three times this dilution was measured in duplicates. ddPCRs contained 8 µl of extraction eluate or diluted spike control and 4 µl of samples drawn within the first 48hrs after surgery.

cfDNA length assessment

The absolute cfDNA values were corrected for the respective spike recovery rate and the amplicon lengths of 98bp and 90bp, either using the size distribution as determined in cfDNA samples from healthy volunteers and patients and the formula given in Figure 2 or using the total cfDNA length index as determined by ddPCR (Figure 3). Simulations were computed for the range of observed size distributions, where the PCR efficiency and the length index were calculated using R (10,000 simulations). The allele frequency distribution, peak 5 to 70%, second and third peak 5 to 45% to cover the observed range of the length index (3 to 30).

Summary and Conclusions

• Given the minute amounts of cell-free DNA, the absolute quantification of GcfDNA requires at first careful control of extraction stage. By adding and quantifying a spiked-in artificial DNA the extraction variability can be corrected.

• For accurate cfDNA quantification a triplet ddPCR assay was developed targeting two genomic loci and the spiked-in DNA. By combining two genomic targets higher numbers of positive droplets are yielded, while keeping the needed sample volume low.

• Since PCR efficiency is lowered by the fragmentation of the cfDNA, a length correction factor was implemented. Individual differences in the cfDNA length distribution profiles introduce only small additional bias which may not be clinically relevant.

• The GcfDNA concentration seems better associated with AST-values early after LTx.

• The initial post LTx phase is characterized by highly variable and high amounts of total cfDNA, which declines with a approximate half life of 1.9 days.

• Absolute GcfDNA quantification may provide a better view on graft integrity in situations, where the host cfDNA is increased due to non-transplantation related causes. Whether the clinical utility improved compared to percentage values is subject to further investigations.

Figure 4: Bland-Altman Plot showing the maximum deviation between the absolute cfDNA values either corrected by the samples experimentally determined length index (n=141, range: 3.7 to 27.5) or by a mean length correction factor of 0.59. The 95% confidence interval indicates that the differences in cfDNA length profiles introduce an error of only 10%.

Figure 5: Comparison of different extraction kits with respect to spike recovery rate (upper panel) and cfDNA yield (middle panel). All extractions were performed on the same plasma pool in triplicate and on three consecutive days, mean values and standard deviations over all extractions are shown. Lower panel: cfDNA yields corrected for the spike recovery rate; no significant differences between the different extraction methods remained, proving the importance and feasibility to correct for extraction efficiency by means of a spiked-in control.

Table 1: Multivariable correlation of GcfDNA percentages and absolute GcfDNA concentration with clinical parameters. Absolute cfDNA yields better correlation with adverse GcfDNA-values significantly correlated to the AST levels, while the percent GcfDNA that do not account for the total cfDNA content per mL of plasma are not. CIT = cold ischemia time, WIT = warm ischemia time, AST = aspartate aminotransferase.