Liquid biopsy for minimally invasive heart transplant monitoring: a pilot study

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ABSTRACT

Background Heart transplantation allows for a long-term management of patients with end-stage heart failure. After the surgery, organ rejection is monitored with endomyocardial biopsy, which is an invasive, but not always informative procedure. Therefore, there is a pressing need for a new, safe, yet reliable, diagnostic method. Here, we present a pilot study confronting liquid biopsy based on donor-specific cell-free DNA with the protocol endomyocardial biopsy.

Methods The study was performed on 21 blood samples matched with endomyocardial biopsy (graded according to acute cellular rejection scale) from nine patients after heart transplantation. Genotyping was performed on genomic DNA from donors and recipients for 10 single-nucleotide polymorphisms (SNPs). Cell-free DNA isolated from plasma was analysed with digital droplet PCR to detect donor-specific alleles.

Results From 21 analysed endomyocardial biopsies, 4 were graded as 0R and 17 as 1R. Liquid biopsy was successfully performed in each sample for all informative SNPs (median of 3 per patient). We observed a high homogeneity of the results between SNPs in each sample (interclass correlation coefficient of >0.9).

Conclusions There is a undeniable need for an alternative, non-invasive diagnostic procedure of early transplant rejection and investigation of donor-derived cell-free DNA seems to be the promising choice. The very high sensitivity is particularly enticing to consider liquid biopsy as a potential screening tool. Its minimal invasiveness may allow for more frequent examination and, thus, tighter monitoring. The reliable assessment of its clinical utility requires an adequately powered and properly designed multicentre study.

INTRODUCTION

Heart transplantation allows for a long-term management of patients with end-stage heart failure. Currently, according to International Society for Heart and Lung Transplantation (ISHLT) reports, heart transplantations are performed in more than 300 centres worldwide, and nearly 6000 hearts were transplanted in 2016.[1] After the surgery, organ rejection is monitored with endomyocardial biopsy. Current state of the art indicates the protocol of nine biopsies during the first year postoperatively.[2] The histological scale for acute cellular rejection (ACR) ranges from 0R to 3R (0R for no lymphocytic infiltrates, 1R for lymphocytic infiltrates with no more than one focus of myocyte damage, 2R for at least two foci of myocyte damage, 3R for diffuse infiltration and severe myocyte damage).[3] Although considered as the golden standard, this method is characterised by several limitations. First, despite employing the minimally invasive endovascular approach, the procedure carries a risk of severe complications (including chorda tendinea rupture or even patient's death).[4 5] Second, although highly specific, the biopsy may not always be informative (due to the scarring after previous biopsies or scarce, non-representative material). Third, the 1R grade seems to be a disproportionately broad category.[6 7]

There is a pressing need for another method of monitoring the transplant rejection, which would be safe, yet reliable. From among the numerous techniques investigated as a potential alternative, cell-free DNA (cfDNA) seems to be the optimal approach, as it may directly reflect the destruction of graft cells. Several strategies of donor-derived cell-free DNA (ddcfDNA) analysis may be employed in terms of both what is investigated and how it is done. In this pilot study, we investigated a panel of 10 single-nucleotide polymorphisms (SNPs) by means of digital droplet PCR (ddPCR). Our aims included: assessment of feasibility and reproducibility, preliminary comparison with endomyocardial biopsy as well as defining the procedures for the subsequent, multicentre study.

MATERIALS AND METHODS

Study group

The study comprised nine patients after heart transplant (eight males and one female; median age: 56 years, range: 40–62). Written informed consent was obtained from each patient. The study was approved by the Bioethical Committee of Medical University of Gdańsk (approval no: NKBBN/138/2018). A total of 21 blood samples matched with endomyocardial biopsy (blood drawn prior to biopsy on the same day) were analysed (1–5 per patient, median: 2).

Endomyocardial biopsy

Endomyocardial biopsy was graded according to ACR grading scale (ISHLT-2004).[3] In addition, ACR grading was subdivided to indicate borderline cases (0/1R was marked in cases with only single infiltrating lymphocytes, while 1/2R was marked in cases with conspicuous lymphocytic infiltrate along with hints of myocyte damage, but not fulfilling 2R criteria). The abundance of lymphocytic infiltrate

Table 1 Summary of SNPs used in the study along with the number of patients for whom the SNP was applicable

SNP ID	Gene	Chromosome	No of patients
rs2942	*GRM1*	chr 6	3
rs4103	*COL4A2*	chr 13	1
rs4331	*ACE*	chr 17	1
rs4523	*TBXA2R*	chr 19	6
rs5182	*AGTR1*	chr 3	4
rs5443	*CDCA3*	chr 12	5
rs5877	*SERPINC1*	chr 1	5
rs9726	*FBXO7*	chr 22	1
rs10966	*ABHD12*	chr 20	6
rs178640	*MYH6*	chr 14	0

SNP, single-nucleotide polymorphism.

was scored semiquantitatively (on the scale of 0 to 5); the presence of granulocytes and Quilty effect was also noted.

Genotyping

For each patient included in the study, genomic DNA (gDNA) was obtained from peripheral blood samples of both graft donor and recipient. gDNA was isolated according to manufacturer's protocol using Wizard Genomic DNA Purification Kit (Promega, Germany). The investigated SNPs were the following: rs2942, rs4103, rs4331, rs4523, rs5182, rs5443, rs5877, rs9726, rs10966 and rs178640 (described in table 1). The genotyping assays were designed by Biogazelle (Belgium). Genotyping was performed with CFX96 Real-Time PCR System (BioRad, USA) using iTaq Universal Probes Supermix (BioRad). For each sample, 10 µL reactions with 20 ng of gDNA were performed in duplicates (initial denaturation at 95°C for 5 min, then 40 cycles of 95°C for 15 s and 55°C for 45 s). The results were analysed with CFX Manager Software (BioRad). Only the informative SNPs for each patient were selected for further analyses.

Liquid biopsy

Peripheral blood samples were obtained prior to endomyocardial biopsy. Blood was drawn into EDTA tubes and stored at +4°C. Within 4 hours plasma was separated by 10 min centrifuging in 1500×*g* followed by 10 min centrifuging in 14 000×*g*. cfDNA was isolated from 1 mL of plasma using QIAamp MinElute ccfDNA Mini Kit (Qiagen, Germany) with the elution volume of 80 µL. ddPCR was performed with QX200 Droplet Digital PCR System (BioRad) using ddPCR Supermix for Probes (BioRad). Droplets were generated from a total reaction volume of 20 µL (including 8 µL of template cfDNA; thus, 10% of the elution volume); the cycling conditions were the following: initial denaturation at 95°C for 10 min, then 50 cycles of 95°C for 30 s and 55°C for 60 s, followed by 98°C for 10 min. For each patient, only the informative SNPs were investigated. For each SNP, a calibration curve (serial dilutions of each allele with 25 ng of gDNA per reaction in total) was performed to assess the analytic validity and optimise detection thresholds. The results were analysed with QuantaSoft software (BioRad).

Statistical analysis

Obtained data were analysed with R (version 3.5.1)[8] with *ggplot2* package for data visualisation.[9] Reproducibility of the results was investigated using interclass correlation coefficient, calculated using the *ICC* package.[10]

Table 2 Summary of histology of classic biopsies

Patient no	Biopsy no	Days since transplant	ACR grade	Subdivided ACR grade	Lymphocytes	Donor allele fraction (%)
1	1	11	1R	0/1R	1	0.08
	2	17	1R	1R	3	0.22
	3	26	1R	1R*	2	0.05
	4	89	1R	1R	1	0.02
2	1	7	1R	0/1R	1	0.19
	2	17	1R	0/1R	1	0.11
	3	28	1R	1/2R	4†	2.36
	4	40	1R	1R	2	0.11
	5	56	1R	1R	3	0.01
3	1	143	1R	0/1R	1	0.05
	2	230	1R	1R	3	0.01
4	1	117	0R	0R	0	0.25
5	1	566	1R	1R	2	0
6	1	295	0R	0R	0	0.04
7	1	8	0R	0R	0	0.03
	2	29	0R	0R	0	0.01
8	1	14	1R	1R	2†	0.15
	2	20	1R	1/2R	4	0.25
	3	28	1R	1R	3	0.29
9	1	13	1R	1R	2	6.62
	2	25	1R	1R	2	0.13

*Indicated the presence of Quilty effect.
†Indicated the presence of granulocytic infiltration.
ACR, acute cellular rejection.

RESULTS

Endomyocardial biopsy

From among the 21 analysed endomyocardial biopsies, 4 were graded as 0R and 17 as 1R; no clear rejection was noted in the study group. Additionally, granulocytes were noted in two cases and Quilty effect in one case. Complete histological data are presented in table 2.

GENOTYPING

Genotyping was performed in all samples. The median number of informative SNPs per patient was 3 (range: 2–5). The median number of patients in which given SNP was informative was 3.5 (range: 0–6).

LIQUID BIOPSY

Liquid biopsy was performed for all informative SNPs in each sample. No significant technical problems were encountered. The results are summarised in table 2 and depicted in figure 1. We observed a high homogeneity of the results between SNPs in each sample (interclass correlation coefficient of 0.955). Elevated ddcfDNA levels were observed in two samples, one of which (2.3) matches with ambiguous histology (dense lymphocytic infiltration, but not fulfilling 2R criterion of two foci of myocyte damage). The other (9.1) was taken from a patient who had undergone cardiac tamponade and cardiac arrest a few days earlier, which might explain the result. The other biopsy with the ambiguous histology (8.2) showed no signs of increased ddcfDNA level; the next biopsy, performed a week later (8.3), showed neither histological nor molecular signs of rejection.

DISCUSSION

Protocol endomyocardial biopsy is currently the golden standard of graft monitoring, however, it is far from being the optimal tool for this purpose. Therefore, numerous biomarkers and

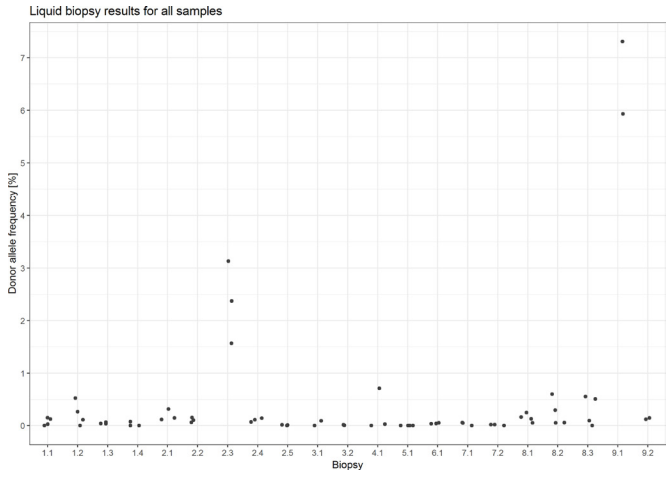

Figure 1 The fractional abundance of donor-derived cell-free DNA.

methodologies have been investigated as a potential alternative.[7 11–13] Analysing ddcfDNA seems to be the optimal choice as it is based on the quantification of single specific molecules released directly from the damaged graft cells. From among the several approaches to distinguish between host and donor DNA, including SNPs,[13] human leucocyte antigen mismatch,[14] InDel differences[15] or copy number variation,[16] we selected SNPs due to their straightforwardness and versatility. The quantification of ddcfDNA was performed with ddPCR, because of its high sensitivity and specificity along with time- and cost-effectiveness sufficient for its implementation in routine diagnostics. With this pilot study, we aimed to assess the feasibility and reproducibility of the method as well as define protocols for the further studies and gather the initial insight into its diagnostic utility.

In terms of reproducibility, we observed high concordance between the results of different informative SNPs in each sample (interclass correlation coefficient value of 0.955), which indicates a good reliability of the method. What is crucial, these data were obtained using a manual droplet generation system, in which the results may be affected to some extent by the technical execution of the procedure. Therefore, the use of automated systems is advisable due to their execution consistency, resulting in the optimal analytic validity (as well as its accurate assessment). In this study, it was not possible to assess the sensitivity and specificity of graft injury detection as there were no samples with clear histological signs of rejection. Nonetheless, it is noteworthy that the results for the majority of samples were consistently low (donor allele fraction <0.5%), while, if observed, the elevation was significant. Three specimens require a further comment. From among the two samples with elevated ddcfDNA levels, one was taken shortly after the patient suffered from cardiac tamponade and cardiac arrest, which might explain the results. The other matched the ambiguous histology with dense lymphocytic infiltration, but no clear rejection. The second sample with borderline endomyocardial biopsy showed no abnormalities in liquid biopsy; however, another sample taken a few days later showed no signs of rejection either in histology or in ddcfDNA. These results advocate for a full evaluation of liquid biopsy in adequately powered multicentre clinical trials.

To date, there is only one report on ddcfDNA analysis by means of ddPCR in heart transplant patients.[13] The study was based on differences in SNPs, similar to our approach, and it was a pilot on 9 kidney, 8 heart and 10 liver transplants.[13] The proportion of observed ddcfDNA in clinically stable heart transplant patients (but not confronted with endomyocardial biopsy) was between 0% and 3.5%,[13] which was similar in our study. This group has recently reported on a study confronting ddcfDNA with creatinine levels and biopsy histology in 189 kidney transplant patients. The donor allele remained below 0.5% or 50 copies/mL in stable patients, while a significant increase was observed on graft injury. What is noteworthy, the authors implemented xenogeneic spike-in to the isolation protocol, thus accounting for isolation efficiency to accurately quantify absolute ddcfDNA levels.[17] A high throughput clinical trial investigating the possibility to monitor heart transplant rejection with liquid biopsy was performed by De Vlaminck *et al*.[11] The study reported on next-generation sequencing of 565 cfDNA samples from 65 transplant recipients. In non-rejecting patients, the donor allele frequencies were very low (tended to 0%), while moderate or severe (2R/3R) rejection was associated with a significant increase (up to 10%), in some cases preceding the diagnosis of rejection by endomyocardial biopsy.[11] On the other hand, the clinical applicability of this method is limited by its high costs and longer turnaround time in contrast to targeted approaches.

Last but not least, it is important to take into account how cfDNA is released into circulation[18]— that is, any cell death (either apoptosis or necrosis) results in DNA release and its subsequent degradation. Therefore, any systemic strain (including an active inflammatory process[19] and even physical effort[20]) leads to a temporary increase in the total cfDNA. Thus, such conditions may affect the fractional abundance of ddcfDNA. On the other hand, the detected amounts of total cfDNA may be affected by numerous technical issues, including sample handling, isolation and reaction preparation.[21] Therefore, reporting both the fractional and total amount of ddcfDNA is crucial, but requires accounting for the cfDNA isolation efficiency with a xenogeneic spike-in as recently described by Oellerich *et al*.[17] Still, it ought to be noted that elevated levels of donor-specific cell-free DNA in plasma may result not only from acute rejection but also from any other type of graft cell injury (ischaemic, toxic, infectious, *etc*.).

To conclude, there is an undeniable need for an alternative, non-invasive diagnostics of early transplant rejection. From among the numerous potential tools, the investigation of ddcfDNA seems to be the promising choice. The very high sensitivity is particularly enticing to consider liquid biopsy as a potential screening tool (with any elevations verified with endomyocardial biopsy), while its minimal invasiveness may allow for more frequent examination and, thus, tighter monitoring. The reliable assessment of its clinical utility requires an adequately powered and properly designed multicentre study.

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