Clinica Chimica Acta 495 (2019) 590–597

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Donor-specific circulating cell free DNA as a noninvasive biomarker of graft injury in heart transplantation

Hada Celicia Macher[a], Noelia García-Fernández[a], Alejandro Adsuar-Gómez[b], Manuel Porras-López[c], Antonio González-Calle[b], José Noval-Padillo[a], Juan Miguel Guerrero[a], Patrocinio Molinero[d], José Miguel Borrego-Domínguez[b], Ángel Herruzo-Avilés[c], Amalia Rubio[d,*]

[a] Dept. of Clinical Biochemistry, Instituto de Investigaciones Biomédicas de Sevilla, IBIS, University of Seville, HUVR, Junta de Andalucía, CSIC, Seville, Spain
[b] Cardiac Surgery Department, Virgen del Rocio University Hospital, Seville, Spain
[c] Intensive Care Unit, Virgen del Rocio University Hospital, Seville, Spain
[d] Dept. Of Medical Biochemistry and Molecular Biology and Immunology, Instituto de Investigaciones Biomédicas de Sevilla, IBIS, University of Seville, HUVR, Junta de Andalucía, CSIC, Seville, Spain

ARTICLE INFO

Keywords:
Hearth transplantation
Graft rejection
Cell-free DNA
Biomarker

ABSTRACT

Background: Considerable effort has been exerted to develop noninvasive diagnostic biomarkers that might replace or reduce the need to perform endomyocardial biopsies. In this context, graft DNA circulating on transplant recipients has been proposed as a potential biomarker of organ rejection or cellular graft injury.
Methods: We propose a digital PCR (dPCR) method based on the amplification of ten specific InDels sufficiently sensitive to detect small amounts of specific donor circulating DNA diluted on the host cell free DNA (cfDNA). We obtained 23 informative mismatches from 30 host and donor organ biopsy pairs.
Results: Patients without heart-related complications showed a high increase in the specific genomic marker levels during the first 24 h after transplantation that dropped to the basal levels on days 3–4 post-surgery. In contrast, patients with complications presented a significantly lagged decay pattern from day one after transplantation. A specific donor cfDNA increase was detected in one patient two days before rejection diagnosis, diminishing the basal levels after successful immunotherapy. A cfDNA increase was also observed during graft injury due to heart damage.
Conclusion: These results suggest that cfDNA monitoring of transplanted patients may be a useful tool to detect and probably anticipate graft rejection.

1. Introduction

Heart transplantation is the gold-standard procedure for end-stage heart failure. Actuarial survival rates in Spain are 76%, 65%, 53% and 25% at 1, 5, 10 and 20 years, respectively [1]. Acute allograft rejection accounts for 10–15% of deaths during the first 3 years [1,2]. Nonetheless, immune injury represents the physiological basis to graft vasculopathy, the leading cause of death during follow up. Monitoring organ rejection is a major clinical concern in the care of heart transplant recipients. Endomyocardial biopsy is the gold-standard technique to evaluate cardiac rejection. It is an invasive procedure, with an estimated risk of 1–2% to predict clinically relevant complications, including death [3]. Furthermore, this method is costly, time-consuming and may be subject to subjectivity and sampling bias.

Considerable effort has been exerted to develop noninvasive diagnostic biomarkers that might replace or reduce the need to perform endomyocardial biopsies. A new approach has gained importance in recent years. Cell free DNA (cfDNA) has been exhaustively studied in recent years as a potential diagnostic, prognostic and monitoring tool in various clinical situations [4–9]. Necrosis or cell apoptosis was first postulated to be the origin of cfDNA, although the exact nature of plasma DNA remains unclear. In this context, graft cfDNA in the circulation of transplant recipients has been proposed as a potential biomarker of organ rejection or cellular graft injury. Lo and coworkers first

Abbreviations: cfDNA, cell free DNA; InDels, insertion-deletion; qPCR, real-time quantitative PCR; dPCR, digital PCR; GE, genome equivalents

* Corresponding author at: Instituto de Biomedicina de Sevilla (IBiS), Campus Hospital Universitario Virgen del Rocio. Avenida Manuel Siurot s/n, planta 2°, lab 210, Seville 41013, Spain.
E-mail addresses: jangle.noval.sspa@juntadeandalucia.es (J. Noval-Padillo), guerrero@us.es (J.M. Guerrero), molinero@us.es (P. Molinero), jmiguel.borrego.sspa@juntadeandalucia.es (J.M. Borrego-Domínguez), amaliarubio@us.es (A. Rubio).

https://doi.org/10.1016/j.cca.2019.06.004
Received 13 March 2019; Received in revised form 14 May 2019; Accepted 4 June 2019
Available online 06 June 2019
0009-8981/

detected chromosome Y-specific sequences in plasma from a female host who received a kidney from a male donor [10]. Since then, several teams have performed different approaches to detect donor-derived specific DNA on host plasma or serum cfDNA.

Initially, research groups had focused their attention on sex-mismatched female recipients of male donor organs, showing that specific sequences from chromosome Y could serve as the donor genetic signature, increasing its levels after an episode of acute rejection [11–15]. Nonetheless, these studies have limitations, referring to a specific subset of patients. Using whole-genome sequencing in all transplanted patients, other groups have demonstrated that the measurement of donor-derived cfDNA not only offers a noninvasive alternative to endomyocardial biopsy but also enables the early diagnosis of graft rejection [16,17].

Trying to expand this promising tool for the entire transplanted population, the main approach would be identifying a genetic organ signature that enables differentiation between the graft cDNA and that of the host. Several insertion-deletion (InDel) diallelic polymorphisms have been described with frequencies higher than 30 in the general population [18]. These InDels may be long sequences or occasionally even complete genes (null alleles). The detection of a donor-recipient DNA mismatch in these sequences is especially useful to monitor organ health after heart transplantation. Thus, the presence of donor cfDNA circulating in a plasma recipient may indicate organ rejection or damage that involves cell death.

In previous work from our group, we analyzed cfDNA in sex-mismatched female recipients of a male donor liver. The SRY gene from chromosome Y was detected by real-time quantitative PCR (qPCR) [15], proving that this noninvasive methodology could be used as a specific marker for this subgroup of patients under liver transplantation. However, qPCR might be not sufficiently sensitive in some cases. Thus, cfDNA may not be detected by this technique when a smaller organ such as the heart is studied. Digital PCR (dPCR) has been proposed as a higher sensitive method to detect the low quantity of DNA target specific sequences. Thus, development of dPCR-based methods may suppose an improved approach for graft cfDNA detection in the host's serum as it has been recently published [19].

In this work, we established a dPCR-based method to detect donor-specific cfDNA present in the recipient's serum after either heart damage or rejection. With this aim, we first determined the donor-recipient DNA mismatch on cardiac transplanted patients to detect the specific signature of all transplanted hearts. Thereafter, we monitored the circulating levels of donor-derived cfDNA in the host's serum during the follow up to associate the cfDNA levels with the clinical evolution of the patients.

2. Materials and methods

2.1. Study subjects

Every consecutive patient who underwent orthotopic heart transplantation at Virgen del Rocio University Hospital (Seville, Spain) between January 2014 and January 2017 and met the inclusion criteria were included in the study. After placement on the waiting list, the patients were informed about the study (unless they were in such a poor clinical status that precluded the individual from understanding the rationale behind the study). After agreement, the patients provided signed informed consent, and they were included in the study. Cases with absent or invalid serum samples at the moment of transplantation were excluded from the study. Our study included 30 heart-transplanted patients. The general characteristics of the patients are shown in Table 1.

2.2. Surgical procedure

During surgery, cardiac surgeons collected tissue samples from both cardiac donors and recipients. Patients from whom either a donor or a host tissue sample could not be collected were excluded from the study.

A blood sample from the central line was collected at two different times during surgery: before extracorporeal circulation and 15 min after reperfusion of the new implanted organ. For circulating DNA determination, 10 mL of blood samples were drawn and centrifuged within 6 h after extraction, and the serum was aliquoted and frozen at −80 °C for future determination.

Table 1
Primer/probe characteristics.

InDel		Primer/probe sequences (5′≥ 3′) [a]	fragment length	Annealing temperature (°C)	
				HRM	dgPCR
GSTM1	Fw	aggaactccctgaaaagctaa	131 nt	50	50
	Rv	gggctcaaatatacggtgga			
	P	FAM-agtttctggggaagcggcca-BBQ			
GSTT	Fw	tccttactggtcctcacatctc	132 nt	52	53
	Rv	ggcagcataagcaggacttc			
	P	FAM- cgtagccatcacggagctgat- BBQ			
SRY	Fw	tggcgattaagtcaaattcgc	127 nt	61	57
	Rv	ccccctagtaccctgacaatgtatt			
	P	FAM-agcagtagagcagtcagggaggcaga-BBQ			
RH (exon-5)	Fw	cgccctcttcttgtggatg	82 nt	60	57
	Rv	gaacacggcattcttcctttc			
	P	HEX- tctggccaagtttcaactctgctcgct-BBQ			
Xq28	Fw	atgggttccaaccagcag	135 nt	55	55
	Rv	aaactgacaattatcacagctt			
	P	FAM- acttgacttgctttccaatggagc - BBQ			
rs4399	Fw	aagcgagtctagtccagc	106 nt	50	48
	Rv	ggaccaggagcacagacaat			
	P	FAM- cctgaaaagcccttgtttctgg - BBQ			
DCP1	Fw	atctcctgacctcgtgatcc	151 nt	59	56
	Rv	ccagcccttagctcacctct			
	P	FAM- ttcgccaattttattccagctct - BBQ			
FVII	Fw	tcccaacttacattcctatatcct	112 nt	58	53
	Rv	cagagcggacggttttgt			
	P	FAM- ttgaagtgttggtgccacacac - BBQ			
R271	Fw	ctcagaggattgactcggg	127 nt	57	58
	Rv	gggggttacgtcttagatgc			
	P	FAM- agactgagcctcgctctcaggc - BBQ			
THYR	Fw	gtcatacaaccaggagaaggtag	107 nt	57	57
	Rv	ccatgttcccaagctctttc			
	P	FAM- caagctggctgacttgatcaca - BBQ			
Control β-globin gene	Fw	gtgcacctgactcctgaggaga	102 nt	61	63
	Rv	ccttgataccaacctgcccag			
	P	FAM-aaggtgaacgtggatgaagttggtgg-BBQ			

[a] The sequences of the primers and probes were designed to distinguish the presence or absence of the InDel. The design was performed to obtain an amplicon ≤150 pb to amplify small cfDNA fragments. When samples were assayed using a commercial Imegen kit, qPCR was performed according to the manufacturer's indications. FW: Forward; Rv: Reverse primers; P: Probe.

2.3. Patient surveillance and treatment

Patients were evaluated clinically and biologically both during their stay in the Intensive Care Unit (ICU) and the ward. Blood samples were drawn by peripheral venipuncture daily during the first three days, and every 2–3 days thereafter. We monitored the circulating DNA in the serum and the standard biochemical, hematological and coagulation parameters, as well as determined the immunosuppressive drug levels. After the patients were discharged, they were followed up at scheduled visits, drawing a blood sample at every visit.

All heart transplant recipients were monitored for acute rejection by scheduled endomyocardial biopsies at 15 days and 1, 2, 3, 4, 6, 9 and 12 months after transplantation and every year thereafter. Nonetheless, in some circumstances, the cardiologist could modify these planned biopsies according to the general status of the patient. The diagnosis of acute rejection was established based on the histopathological findings on graft biopsy according to the revised nomenclature for graft

rejection by Stewart et al. [20]

The cohort patients were treated with antiviral prophylaxis and immunosuppression in standardized posttransplant therapy:

a) Induction therapy during the first 48 h (thymoglobulin in high-immunological-risk patients and anti-interleukin-2 receptor antibodies—daclizumab and basiliximab—in the others).
b) Maintenance therapy: starting 24 h after transplantation, it was based on 3 cornerstone immunosuppressants: steroids, calcineurin inhibitors (cyclosporine or tacrolimus) and anti-proliferative agents (mycophenolate or, more recently, mTOR inhibitors everolimus and rapamycin).

2.4. Ethics statement

Ethical approval for the study protocol was granted by the Medical Research Ethics Board of the Virgen del Rocío University Hospital of Seville. The clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki.

2.5. General workflow

We proposed an approach to detect donor-derived cfDNA in the recipient based on the existence of long genomic sequences (InDels), which are either present or absent in the individual genotype. A panel of ten InDels previously described by Jimenez-Velasco [18] was selected to differentiate donor cfDNA from that of the recipient. This panel consists of four null alleles (GSTM1, GSTT1, SRY and RhD) and six InDels (DCP1, Xq28, R271, rs4399, FVII and THYR). The chromosome position, length of the insertion sequence and frequency of the different InDels are shown in Table 2. Several samples without a genomic signature by the InDels proposed were assayed using a commercial kit (Imegen® Quimera Screening Multiplex Plus kit; Imegen, Valencia, Spain).

We were interested in evaluating the early dynamics of graft injury and recovery. The workflow for this objective involves two steps as follows. The first step comprised InDel identification by qPCR. Tissue samples from both donors and hosts were analyzed to determine a donor-recipient mismatch for the InDels analyzed. A mismatch was considered informative when a deleted sequence on the host DNA, but not in the donor DNA, was observed. The second step consists of the quantification of donor DNA in the recipient's serum. After the detection of an informative mismatch, cfDNA from sequential serum samples was analyzed by dPCR. The presence of elevated donor cfDNA circulating in serum from the recipient will be indicative of any type of transplanted heart complication. We also quantified beta-globin gene values as a control for general damage or patient clinical worsening by qPCR assay.

Table 2
General characteristic of the patients.

		(n = 30)
Age	Median [range]	54.5 [30–70]
Sex	Male	22 (73.3%)
Ischemia time (min)	Media ± SEM	209.62 ± 10.38
Pump time (min)	Media ± SEM	130.54 ± 7.28
Exitus[a]	Total	4 (13.3%)
Time until exitus (days)	Median [range]	75.25 [57–94]
Previous ICU		10 (33.3%)
Previous ICU time (days)	Median [range]	7.5 [2–12]
ICU post-transplantation (days)	Median [range]	6.5 [3–89]
Hospitalization (days)	Median [range]	24 [7–94]
Rejection	No rejection (0 R)	17 (56.6%)
	Mild rejection (1–2 R)	10 (33.3%)
	Severe (3 R)	3 (10%)

[a] Causes of death: three patients died due to multiple organ failure as a consequence of either shock septic (*Klebsiella pneumoniae*), or after a long period at the ICU with severe complications; the fourth patient died due to ischemic cardiogenic shock.

2.6. DNA extraction from tissue and serum samples

DNA from tissue samples was extracted using QIAcube (Qiagen GmbH, Germany), according to the manufacturer's protocol. Five grams of donor and host biopsy samples were previously minced and incubated with 180 µL of ATL lysis buffer (animal tissue lysis buffer) and 20 µL of proteinase K for 2 h at 56 °C. The DNA was eluted in a final volume of 100 µL and was frozen at −80 °C until determination.

DNA from 400 µL of serum samples was extracted using the automatized MagNaPure Compact Instrument (Roche Diagnostics, Basel, Switzerland) using the Magna Pure Compact Nucleic Acid Isolation Kit I, according to the protocol "Total NA Plasma 100 400 V3 1". The DNA was eluted in a final volume of 50 µL and was frozen at −80 °C until either qPCR or dPCR.

Quantification of the nucleic acids after DNA isolation was performed using Qubit 3.0 fluorometry (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.7. Determination of donor-recipient mismatch

Selected InDels were amplified from donor and host tissue DNA by qPCR assay using the Light-Cycler 480 Real-Time PCR instrument (Roche Diagnostics, Basel, Switzerland). Two microliters of DNA were amplified in a final volume of 20 µL containing 200 nM of primers and 100 nM of the probe using theLC480 ProbesMaster Kit (Roche Diagnostics, Basel, Switzerland). qPCR was performed at 95 °C for 5″ and at the specific InDel Tm for 20″ for 40 cycles. The standards for the calibration curve were based on the dilutions of human genomic DNA (Roche Diagnostics, Basel, Switzerland). The primer and probe sequences, as well as experimental conditions, are shown in Table 2.

2.8. Digital PCR

dPCR assays were performed using the QX200 Droplet DPCR System (Bio-Rad, Hercules, CA, United States). DNA from human genomic DNA (Roche Diagnostics GmbH, Mannheim, Germany) was used as a positive control. Background amplification was measured by adding water to the reaction mixture instead of DNA. All samples were analyzed in duplicate. The PCR primers and probes are detailed in Table 2. The PCR assay mixture (20 µL) contained 10 µL of dPCR Supermix (no dUTP) for probes, 0.5 µL of each primer, 0.6 µL of probe labeled with FAM fluorophores, and 8.4 µL of serum-extracted DNA. The amplicon sizes ranged between 106 and 151 bp. Thermal cycling consisted of 10 min at 95 °C, followed by 48 cycles of 95 °C for 20 s, specific amplicon temperature for 30 s (Table 2), and 64.5 °C for 15 s, and finally 10 min at 98 °C. The PCR settings were based on a manually performed temperature gradient.

Sample analysis of each experiment was performed using QuantaSoft v1.7 software (Bio-Rad Laboratories, Hercules, CA, USA). Positive droplet concentrations in all samples were determined using manually placed fluorescence thresholds based on negative clusters as detected in the corresponding NTCs. The target DNA concentration (copies/µL) and absolute droplet counts within single samples were used as the quantitative outcome measurement. All samples and controls were assayed in duplicate. The final concentration was calculated according to the following formula:

$$c = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}}$$

where Q represents the total number of DNA copies, V_{DNA} represents the total volume of cfDNA obtained after extraction from serum, V_{PCR} represents the sample volume used for PCR, and V_{ext} represents the

volume of extracted serum.

2.9. Determination of the beta-globin gene

We also quantified the circulating serum beta-globin gene as a control for general clinical worsening by qPCR using a Light-Cycler 480 Real-Time PCR instrument (Roche Diagnostics, Basel, Switzerland) as previously described [15]. We considered levels over 500 ng/μl indicative of patient suffering any kind of clinical complication (mean control levels 356 ± 76.05 ng/μl). A conversion factor of 6.6 pg of DNA per diploid cell was used to express the cfDNA concentration as genome equivalents (GE). The same formula described above was used to determine the KGE/mL of serum.

3. Results

3.1. Characteristic of the patients

The general characteristics of the patients are shown in Table 1. Most were males (73.3%), and the age ranged from 30 to 70 years. Ten patients were at the ICU previously for the transplantation procedure with a mean duration of 1 week. After surgery, all the patients stayed at the ICU for various durations (3 to 89 days) depending on the general outcome of the patient. Only 4 patients (13.3%) died during the follow up. None of them died because of graft rejection. The causes of death were as follows: three patients died due to multiple organ failure as a consequence of either shock septic (*Klebsiella pneumoniae*), or after a long period at the ICU with severe complications; the fourth patient died due to ischemic cardiogenic shock. Two of these patients (patients 16 and 23) were not evaluated during the follow up because we found no valid informative InDel. Severe graft rejection was reported in 3 patients (10%), who, in all cases, were controlled after treatment adjustment. In two cases, organ rejection appeared approximately six to seven months after patient discharge, and a serum sample was not available for determination.

3.2. Determination of donor-recipient mismatch

We obtained 23 (76.6%) informative mismatches from the 30 hosts and donor organ biopsy pairs. Four hosts (13.3%) were women who received a male organ transplant, and the SRY gene was useful as an informative genomic marker. The individual presence/absence of the other markers and InDel frequency and informativeness are shown in Table 3. Two patients with no informative mismatch could be monitored using the Imegen kit (29D and 12D markers). Patient 7 was monitored using the Imegen 4 l marker in addition to the specific one.

3.3. Monitoring of donor organ health by dPCR

Most of the patients (23 patients) were evaluated during the follow up. Among these 23 patients, 18 had successful heart transplantation with no or mild organ rejection and no additional heart-related complications during the recovery at the ICU. All 18 patients showed a high increase in specific genomic marker levels during the first 24 h after transplantation that was dramatically reduced to basal levels on days 3–4 post surgery (range 58–72 copies/ml serum sample) and was undetectable after one week in most patients (Fig. 1A). The mean ± standard error of the measurement of the marker of interest during normal function distinct from rejection was 15.62 ± 2.59 copies/mL of serum sample (median and range 1.5 [0–68.75] copies/mL).

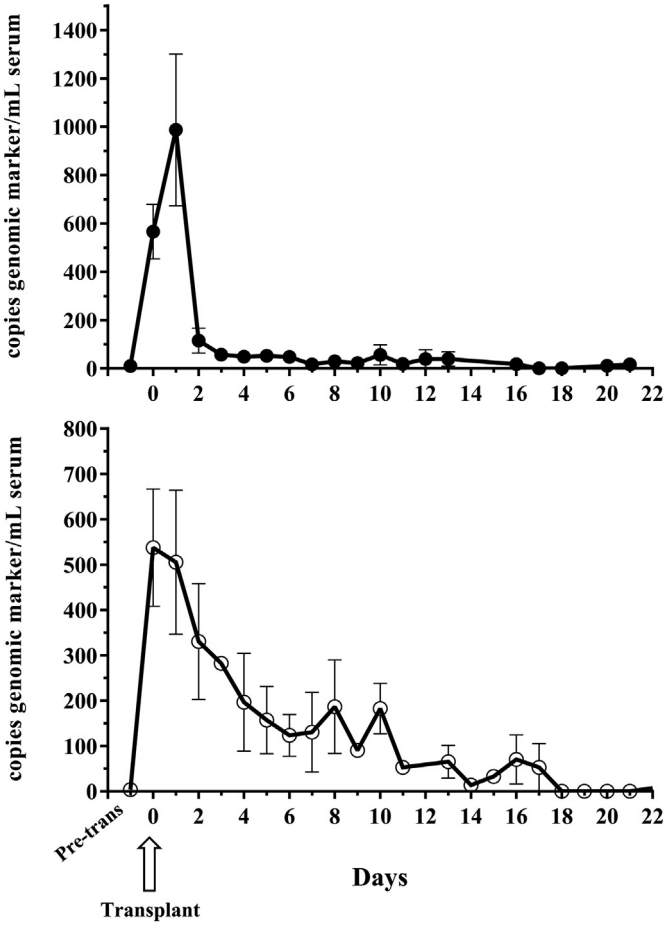

Fig. 1. Copies of the specific genomic marker. Mean number of copies of serum-specific cfDNA levels from A) patients who accepted transplanted hearths without any complications and B) patients with any type of post-transplantation complication during the first three weeks after transplantation (mean ± SEM). Pre-trans: pre-transplantation sample before organ reperfusion.

Table 3
Informative mismatch frequency (fq).

	GenBank accession	Deleted Homozygote fq	Deleted homozygote fq found		Mismatches found (%)	Informative Fq found
			Host	Donor		
GSTM1	NT_019273	47%	17% - 13.3%		7 (23.3%)	4 (13.3%)
GSTT1	AB057594	17%	6.6% - 6.6%		4 (13.3%)	2 (6.6%)
Xq28	AF003626	30%	10% - 0%		3 (10%)	3 (10%)
rs4399	AL008720	16%	60% - 43.3%		8 (26.6%)	6 (20%)
DCP1	X62855	32	30% - 30%		12 (40%)	6 (20%)
FVII	J02933	63%	36.6% - 23.3%		4 (13.3%)	5 (16.6%)
R271	AC009286	25%	3.3% - 0%		1 (20%)	1 (3.3%)
THYR	AY053519	22%	3.3% - 6.6%		3 (10%)	1 (3.3%)
RHD	AF187846	10%	13.3%-13.3%		8 (26.6%)	4 (13.3%)

Four host (13.3%) were women that received a male organ transplant and SRY gene was useful as an informative genomic marker.

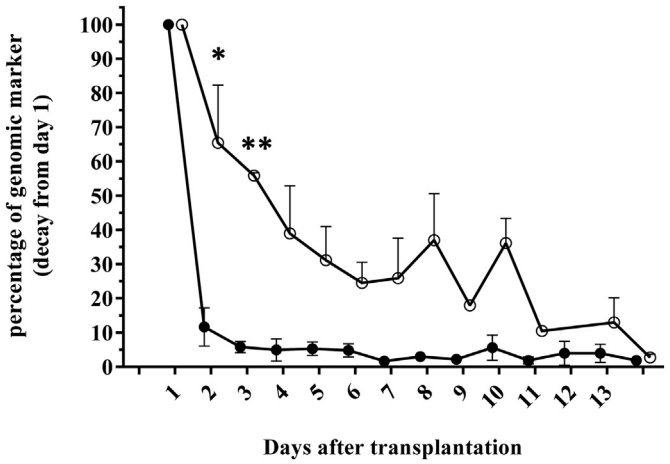

Fig. 2. Comparison of the serum percentage of the decrease in the specific genomic marker levels in patients with and without complications. Comparison of the decay patterns in patients with and without complications expressed as the diminution percentage after the maximal peak values obtained at day 1 after transplantation. *t*-test, (*) $p < 0.05$ and (**) $p < 0.0001$.

By contrast, patients with complications presented elevated specific marker levels during the first week, as shown in Fig. 1B (130 copies/mL at day 7). These complications include a transient heart rejection, respiratory infections, renal dysfunction and ventricular dysfunction after surgery. Additionally, the dramatic decay of cfDNA during the first 48 h observed in patients with good evolution was not observed in patients with complications. Fig. 2 shows different decay patterns in both groups of patients expressed as the mean diminution percentage after the maximal peak values obtained on day 1 after transplantation. Significant differences were found at days two and three comparing patients with good evolution and patients with any type of graft complication [*t*-test; 12% versus 65% decrease at day 2 ($p < 0.05$) and 6% versus 56% decrease at day 3 ($p < 0.0001$)].

Analyzing InDels' sensitivity by dPCR we have observed some differences among them. Thus, mean peak values of RH and 28× markers were > 1000 copies/ml; DCP, res4329 and IMEGEN markers were between 500 and 1000 copies/mL; and SRY, r271 and GSTM markers were < 500 copies/mL.

3.4. Individual analysis of patients with complications

3.4.1. Patients with heart rejection

One patient (patient 7) displayed two severe transient heart rejections 10 days and 1 month after surgery, respectively. Treatment adjustment eventually enabled organ tolerance by the patient. This patient showed an informative mismatch for DCP InDel that was used as a genomic biomarker. To confirm the organ rejection, we also analyzed cfDNA from the patient using the commercial Imegen kit (InDel 4l). Additionally, this patient was a woman who received an organ from a

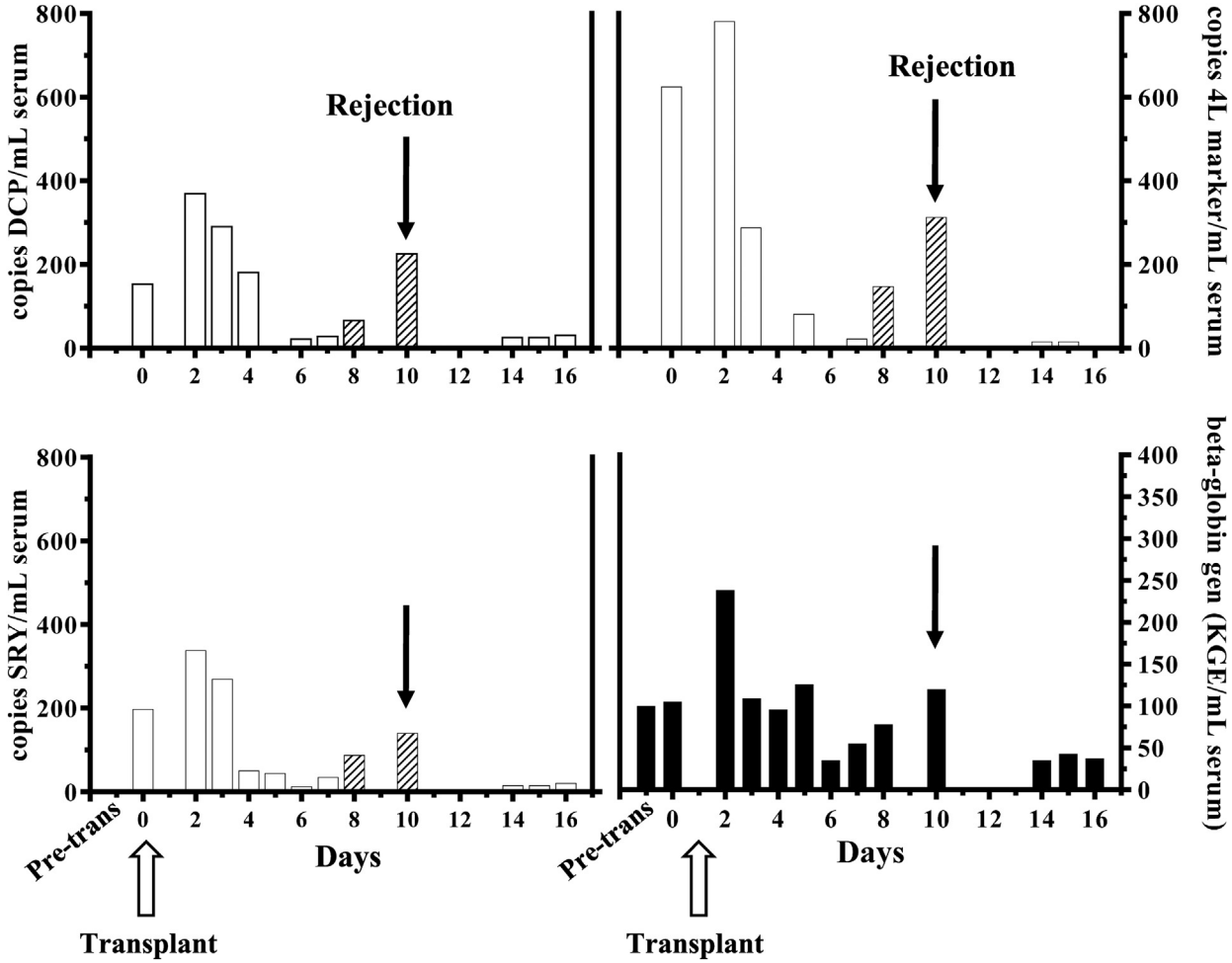

Fig. 3. Serum-specific markers and beta-globin gene cfDNA copies of patient 7. Copies of three different specific markers, A) DCP, B) SRY gene and C) 4 L (Imegen), as well as the D) beta-globin levels during the follow up. Confirmation of rejection during this period is indicated by an arrow. Pre-trans: pre-transplantation sample before organ reperfusion. White bars: specific InDels levels; black bars: beta-globin levels; striped bars transient rejection.

male donor, and SRY gene amplification was also determined as an additional genomic marker. Fig. 3 shows that levels of all genomic markers analyzed presented a similar pattern. Thus, genomic marker levels were highly increased during the first to third days after transplantation, were decreased on days four to five, and were almost undetectable after one week. On day 8, the cfDNA levels started rising over basal levels, and a clear increase was observed on day 10 when organ rejection was diagnosed by endomyocardial biopsy. A second heart rejection was repeated after 20 days; however, unfortunately, no sample was available at this time-point. However, we could assay DCP levels on days 17 and 18 after transplantation. A high increase was observed on days 17 and 18, with the copy levels on day 18 higher than double those found during rejection on day 10 (418.75 versus 225 copies/mL of serum). Sufficient samples for SRY and Imegen kit analyses were not available at these last time-points. Regarding the beta-globin levels, Fig. 3D shows a decreased level after surgery and a single point increase on day 10 coincident with that of the other specific markers. The mean beta-globin values of all patients included are shown in supplemental material (Supplementary Fig. S1). The mean values were considerably variable and did not diminish until the basal levels during the first month of follow up.

3.4.2. Patients with serious complications after heart transplantation

Three patients went through some kind of heart complication episode, and we could examine them during the follow up. Two patients had serious complications after the heart transplantation, patient 6 and patient 26. Both patients showed only one informative mismatch for DCP InDel. Fig. 4A shows the DCP levels of patient 6 evolution. This patient was a 70 years old patient with poor evolution after transplantation (*Klebsiella pneumoniae* respiratory infection, renal dysfunction, and hemodynamic instability) and was at the ICU during the whole follow-up period. After day 66, a general worsening of the patient was observed with a clinical scenario of sepsis secondary to tracheobronchitis by *Stenotrophomonas maltophilia*. Finally, after an extremely long period at the ICU, the patient died due to multiorgan failure three months after transplantation. Although the DCP levels had decreased until undetectable levels after two weeks, the beta-globin levels were variable but elevated during the complete follow up of the patient. Higher values were observed during the last two months, suggesting general deterioration of the patient's health. A transient peak of DCP levels (84.37 copies/mL serum) was also observed at week 65 coincident with an increase in the beta-globin levels (133.65 KEG/mL) and a general poor evolution of the patient during these weeks. Patient 26, transplanted due to a cardiogenic shock, spent 21 days at ICU before surgery with a severe respiratory infection. This patient presented a bad scenario after surgery suffering a septic shock, metabolic acidosis and brain affectation by anoxic encephalopathy. During the first weeks, antibiotherapy and suboptimum immunosuppressive therapy were administered. After two weeks, antibiotherapy treatment was suspended, tacrolimus dose was risen up to optimal levels, and finally, the patient shown a favorable outcome one month after transplantation. This patient, shows a similar cfDNA pattern during the first days after transplantation (Fig. 4B), with elevated DCP levels during the first two weeks (> 100 copies/mL) and very high beta-globin levels (171.04 KGE/mL at day 14). However, after one month, not only the DCP levels became undetectable but also the beta-globin levels were diminished until the patient was discharged at day 57 after transplantation.

Patient 19 underwent complicated surgery. He presented left ventricular dysfunction with cardiocirculatory support dependence during the first two weeks after transplantation (Fig. 5). The levels of the specific marker were extremely high during the first five days after transplantation. As ventricular dysfunction persisted, on day 11, the patient was included again in the transplant priority list for a second heart transplant. The patient cfDNA was monitored during the first transplantation and two weeks after the second one (second transplantation on day 15). We found no informative InDel for the second

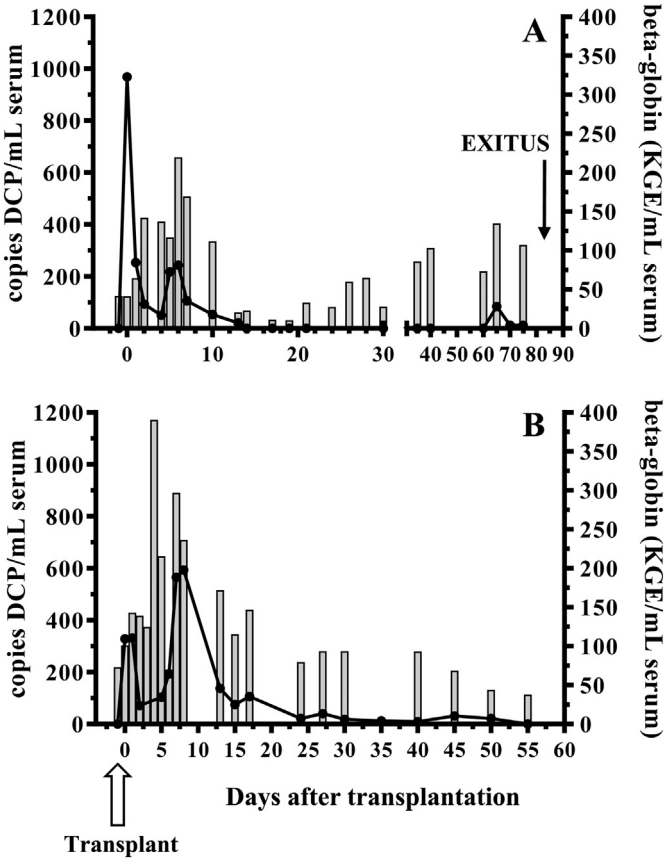

Fig. 4. Profile of specific marker (DCP) and beta-globin gene cfDNA levels of two patients with severe complications after transplantation. DCP and beta-globin levels during the evolution of A) a patient with severe complications after transplantation who died at day 90 (Patient 6), and B) a patient with initial poor evolution who was finally discharged on day 57 (patient 26). Black line: specific InDel levels; bars: beta-globin levels.

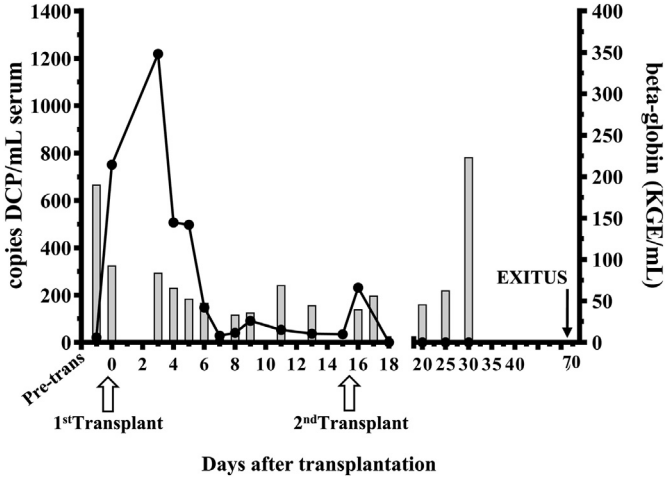

Fig. 5. Profile of the specific marker (DCP) and beta-globin gene cfDNA levels of patient 19. The DCP and beta-globin levels during the evolution of a patient who developed myocardial ischemic injury and was retransplanted on day 15. Patient 19 had severe complications during the complete follow up and died on day 72. Black line: specific InDel levels; bars: beta-globin levels.

transplant. As expected, after retransplantation, the specific InDel levels for this patient (DCP) became undetectable, although the beta-globin levels continued increasing during the next two weeks of the second transplant. Although the second transplant was successful, the general

Table 4
Follow up of patient 15 after heart transplantation.

Days	29D marker (copies/μL)	Beta-globin (KGE/mμL)	Tacrolimus (ng/mL)	Troponin (ng/L)	CPK (mU/mL)	Creatinine (mg/dL)	CRP (mg/L)
Pre-T	0	61.25	nd	[a]510.6	[a]351	[a]0.32	[a]162.5
0	750.00	6.31	nd	[a]510.6	[a]351	[a]0.32	[a]162.5
2	53.12	41.04	nd	[a]412.4	151	[a]0.43	[a]171.8
4	25.00	55.01	nd	nd	71	nd	[a]88.9
5	12.5	37.29	2.4	[a]383.2	39	[a]0.49	[a]73.5
8	25.00	25.20	9.7	[a]355.3	35	[a]0.47	[a]58.3
14	20.00	15.85	4.3	[a]313.1	[a]18	0.74	[a]79.7
16	103.12	15.64	6.5	[a]235.9	[a]20	0.77	[a]49.4
19	21.87	20.02	5.7	[a]247.3	[a]18	0.67	[a]30.9
21	**43,500.00**	50.41	**< 1**[b]	[a]258.3	[a]17	0.79	[a]31.0
23	31.25	4.68	9	[a]273	31	0.82	[a]28.6
30	31.25	9.20	5	nd	nd	1.02	nd

(nd) non-determined.
Bold: Significant increased in of specific cfDNA and undetectable levels of Tacrolimus at day 21.
[a] Values out of normal clinical range.
[b] Values lower than 1 ng/mL, non-detectable by the technique.

outcome of the patient was worsening. During the long period at the ICU, he developed *K. pneumoniae* infection, renal dysfunction and severe thrombocytopenia. Finally, after 73 days at the ICU, he died two months after the first transplantation due to multiorgan failure.

3.4.3. Patient with transient poor adherence to the treatment

Patient 15 had a different evolution from the ones described above. This patient had a good evolution and no sign of rejection during the follow up. The specific genomic marker (29D from the Imegen kit) levels were rapidly diminished after transplantation and continued at basal levels during the period studied, except for one time point, day 21 after transplantation (Table 4). On that day, the 29D marker levels were extremely increased compared with those of the sample assayed two days prior (43,500 versus 21.87 copies/mL of serum) and returned to normal levels two days later. Other clinical and biochemical parameters were also analyzed. As illustrated in Table 4, at this time-point, most of the parameters studied were similar to the surrounding days except the genomic marker levels (beta-globin and 29D marker) and immunosuppressor drug levels (tacrolimus), which were undetectable on this day. After correcting the immunosuppressor dose, the plasma levels of tacrolimus returned to the normal range in two days, coincident with the decrease in the 29D genomic marker.

4. Discussion

Cell death has been generally accepted to be an important cause of the release of DNA into the plasma [6]. In the context of heart transplantation where different genetic material is present in the host, this distinct DNA might be found circulating in the blood after either heart damage or rejection. In this work, transplanted heart health was analyzed using a dPCR-based method. Specific donor cfDNA increase was detected previously and during heart complication due to either rejection or heart damage.

Although endomyocardial biopsy continues to be the gold standard to detect cardiac rejection, it is an invasive procedure not exempt from risk. Additionally, this technique may be prone to subjectivity due to inter-observer variability in grading heart biopsies. This issue was addressed in the CARGO II study, where the concordance of biopsy interpretation among different cardiac pathologists was evaluated [21]. The authors concluded that an endomyocardial biopsy grade higher than or equal to 2R is not by itself sufficient for clinical decisions or as a research criterion. Furthermore, this method provides information on single spots of myocardial tissue. Thus, considering graft damage is not homogeneous, acute rejection might be missed.

Considerable effort has been exerted to develop noninvasive techniques that might replace or reduce the need to perform heart biopsies. We have developed a dPCR method to quantify low amounts of donor-derived cfDNA from the sera of patients after heart transplantation. This method is based on the amplification of donor-specific InDels being sufficiently sensitive to detect small amounts of specific donor cfDNA diluted on the host cfDNA. Thus, the beta-globin levels were 10^2 higher than the maximal mean of the specific InDel. We also observed that the amplification may differ depending on the InDel analyzed, resulting in some variations and suggesting that primer/probe systems for certain InDels may be more sensitive than others. Using the test InDel panel proposed, we found that most of the patients from our population could be monitored after transplantation being the frequency and informativeness of the different InDels variable.

Complications observed during the stay at the ICU resulted in elevated specific marker levels during this period. Analysis of cfDNA decay during the first two weeks suggests that graft cfDNA evaluation during the first weeks after transplantation may provide useful information not only concerning organ rejection but also concerning any type of surgery-derived complication involving cell death. However, further studies including a higher number of patients should be performed to confirm these data.

Only one patient exhibited severe heart transient rejection during the observational period. The three specific InDels analyzed showed an increase from undetectable levels two days before rejection, rising maximal levels on rejection reported by endomyocardial biopsy and decreasing thereafter due to treatment adjustment. Even though only one patient showed a heart rejection during the observational period, these results suggest that cfDNA monitoring of transplanted patients might be a useful tool to detect and probably anticipate graft rejection. In this way, other authors have also described that donor cfDNA elevations can occur before heart rejection and may present an opportunity for early diagnosis and treatment [17]. Furthermore, the rapid decrease after the appropriate treatment might allow the monitoring of the early response to immunosuppressive drugs.

Patients without rejection but with severe complications after heart transplantation showed a similar pattern during the follow up. Although donor cfDNA was diminished after several weeks, the beta-globin gene levels only decreased after patient stabilization and discharge from the ICU. By contrast, patients with poor evolution maintained elevated beta-globin gene levels, suggesting a general deterioration of the patient health. These trends were illustrated in patients 6 and 19 who had finally died. Elevation of the beta-globin gene levels, which is present in both host and donor cfDNA, may be due to a general cell damage associated with a clinical worsening of patients' health. Thus, we consider that a constitutive gene, such as the beta-globin gene, should always be evaluated along with specific markers.

One patient with good evolution (patient 15) caught our attention

due to an extremely high level of cfDNA at a single time point, coincident with undetectable immunosuppressive drug levels. We speculate that the single-point poor adherence to the treatment may have caused the transient increase in the specific genomic marker. It has been described that donor cfDNA may be informative of the therapeutic ranges of the tacrolimus concentrations after liver transplantation [22]. Thus, they described a significant relationship between low tacrolimus levels and donor-specific cfDNA ≥10% of the total cfDNA. In this paper, the authors suggest that graft evaluation by donor cfDNA determination might be especially useful to detect graft injury during immunosuppressant dose-minimization strategies.

Different methods have been proposed to quantify increases in donor-derived cfDNA on host plasma. The detection of several donor cfDNA-specific polymorphisms has been proposed, such as shotgun sequencing [16,17], target sequencing [23], or dPCR [19]. However, most deep sequencing-based methods are time-consuming to be clinically useful for patient monitoring. In the present work, we have developed a dPCR-based method that can detect increased specific donor cfDNA due to heart complication after transplantation. Following a similar approach, Adamek et al. proposed a method based on real-time PCR followed by gel analysis of the amplicon length by amplification of specific InDels. They validated this technique in eight kidney-transplanted patients during the first 3 days after transplantation. We believe that more sensitive techniques such as dPCR are necessary for donor-specific cfDNA to monitor heart transplantation. It has been described that different organs might release different amounts of cfDNA [24]. Thus, we could quantify donor cfDNA in liver-transplanted patients [15] but not in heart-transplanted ones. Analyzing liver- and kidney-transplanted patients, Beck [19] et al. described an interesting method based on the detection of SNPs by dPCR. They identified both the early rejection and vascular causes of graft damage by the quantification of graft-derived cfDNA as a percentage of total DNA. Different approaches described until now have suggested that donor-specific cfDNA measurement may be an interesting approach in the clinical evaluation of organ transplant. The test described herewith involves a rapid turnaround process that may allow the adoption of clinical decisions about patient evolution. Further research should be performed to increase the number of patients to validate the approach proposed.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.06.004.

Acknowledgments

We thank Pilar Jimenez-Arriscado for her disinterested help in the achievement of this work.

Funding

This work was supported by a grant from the Instituto de Salud Carlos III [FIS: PI15/00939]-Fondos FEDER. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. Authors declare no conflicts of interest and no involvement of a commercial organization in manuscript preparation or funding.

References

[1] F. González-Vilchez, J. Segovia-Cubero, L. Almenar, et al., Spanish heart transplantation registry. 27th official report of the Spanish Society of cardiology working group on heart failure and heart transplantation (1984–2015), Rev. Esp. Cardiol. 69 (11) (2016) 1071–1082.
[2] K.K. Khush, W.S. Cherikh, D.C. Chambers, et al., The international thoracic organ transplant registry of the International Society for Heart and Lung Transplantation: thirty-fifth adult heart transplantation report 2018; focus theme: multiorgan transplantation, J. Heart Lung Transplant. 37 (10) (2018) 1155–1168.
[3] K.S. Cunningham, J.P. Veinot, J. Butany, An approach to endomyocardial biopsy interpretation, J. Clin. Pathol. 59 (2) (2006) 121–129.
[4] Y.K. Tong, Y.M. Lo, Diagnostic developments involving cell-free (circulating) nucleic acids, Clin. Chim. Acta 363 (1–2) (2006) 187–196.
[5] S.A. Leon, B. Shapiro, D.M. Sklaroff, et al., Free DNA in the serum of cancer patients and the effect of therapy, Cancer Res. 37 (3) (1977) 646–650.
[6] R.W. Chiu, Y.M. Lo, Noninvasive prenatal diagnosis by analysis of fetal DNA in maternal plasma, Methods Mol. Biol. 336 (2006) 101–109.
[7] R.W. Chiu, Y.M. Lo, Clinical applications of maternal plasma fetal DNA analysis: translating the fruits of 15 years of research, Clin. Chem. Lab. Med. 51 (1) (2013) 197–204, https://doi.org/10.1515/cclm-2012-0601.
[8] H.C. Macher, P. Noguerol, P. Medrano-Campillo, et al., Standardization non-invasive fetal RHD and SRY determination into clinical routine using a new multiplex RT-PCR assay for fetal cell-free DNA in pregnant women plasma: results in clinical benefits and cost saving, Clin. Chim. Acta 413 (3–4) (2012) 490–494.
[9] H.C. Macher, M.A. Martinez-Broca, A. Rubio-Calvo, et al., Non-invasive prenatal diagnosis of multiple endocrine neoplasia type 2A using COLD-PCR combined with HRM genotyping analysis from maternal serum, PLoS ONE 7 (12) (2012) e51024.
[10] Y.M.D. Lo, M.S.C. Tein, C.C.P. Pang, et al., Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients, Lancet 351 (9112) (1998) 1329–1330.
[11] J. Zhang, K.L. Tong, P.K. Li, et al., Presence of donor- and recipient-derived DNA in cell-free urine samples of renal transplantation recipients: urinary DNA chimerism, Clin. Chem. 45 (10) (1999) 1741–1746.
[12] Z. Zhang, N. Ohkohchi, H. Okazaki, et al., Use of PCR and PCR-SSP for detection of urinary donor-origin DNA in renal transplant recipients with acute rejection, Chin. Med. J. 116 (2) (2003) 191–194 (Engl).
[13] X.Y. Zhong, D. Hahn, C. Troeger, et al., Cell-free DNA in urine: a marker for kidney graft rejection, but not for prenatal diagnosis? Ann. N. Y. Acad. Sci. 945 (2001) 250–257.
[14] V. García Moreira, B. Prieto García, J.M. Baltar Martín, et al., Cell-free DNA as a noninvasive acute rejection marker in renal transplantation, Clin. Chem. 55 (11) (2009) 1958–1966.
[15] H.C. Macher, G. Suarez-Artacho, J.M. Guerrero, et al., Monitoring of transplanted liver health by quantification of organ-specific genomic marker in circulating DNA from receptor, PLoS ONE 9 (12) (2014) e113987.
[16] T.M. Snyder, K.K. Khush, H.A. Valantine, et al., Universal noninvasive detection of solid organ transplant rejection, Proc. Natl. Acad. Sci. U. S. A. 108 (15) (2011) 6229–6234.
[17] I. De Vlaminck, H.A. Valantine, T.M. Snyder, et al., Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection, Sci. Transl. Med. 6 (241) (2014) 241ra77.
[18] A. Jiménez-Velasco, M. Barrios, J. Román-Gómez, et al., Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms, Leukemia 19 (3) (2005) 336–343.
[19] J. Beck, S. Bierau, S. Balzer, et al., Digital droplet PCR for rapid quantification of donor DNA in the circulation of transplant recipients as a potential universal biomarker of graft injury, Clin. Chem. 59 (12) (2013) 1732–1741.
[20] S. Stewart, G.L. Winters, M.C. Fishbein, et al., Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection, J. Heart Lung Transplant. Nov 24 (11) (2005) 1710–1720.
[21] M.G. Crespo-Leiro, A. Zuckermann, C. Bara, et al., Concordance among pathologists in the second cardiac allograft rejection gene expression observational study (CARGO II), Transplantation 94 (2012) 1172–1177.
[22] M. Oellerich, E. Schütz, P. Kanzow, et al., Use of graft-derived cell-free DNA as an organ integrity biomarker to reexamine effective tacrolimus trough concentrations after liver transplantation, Ther. Drug Monit. 36 (2) (2014) 136–140.
[23] M. Hidestrand, A. Tomita-Mitchell, P.M. Hidestrand, et al., Highly sensitive noninvasive cardiac transplant rejection monitoring using targeted quantification of donor-specific cell-free deoxyribonucleic acid, J. Am. Coll. Cardiol. 63 (2014) 1224–1226.
[24] Y.Y.N. Lui, K.S. Woo, A.Y.M. Wang, et al., Origin of plasma cell-free DNA after solid organ transplantation, Clin. Chem. 49 (2003) 495–496.