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ORIGINAL ARTICLE

Identification of a gene expression profile associated with operational tolerance among a selected group of stable kidney transplant patients

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Summary

Despite their utility, immunosuppressive treatments have numerous side effects, including infectious complications, malignancies and metabolic disorders, all of which contribute to long-term graft loss. In addition to the development of new pharmaceutical products with reduced toxicity and more comfortable modes of administration, tailoring immunosuppression according to the immune status of each patient would represent a significant breakthrough. Gene expression profiling has been shown to be a clinically relevant monitoring tool. In this paper, we have assessed the overall long-term kidney transplant outcome and attempted to identify operationally tolerant-like patients among recipients with stable clinical status at least 5 years post-transplantation. We thus measured a combination of noninvasive blood biomarkers of operational tolerance in a cohort of 144 stable patients and showed that only 3.5% exhibited a gene expression profile of operational tolerance, suggesting that such a profile can be detected under immunosuppressive therapy but that its frequency is low in kidney transplant recipients when compared with liver transplant recipients. We suggest that a rational approach to patient selection, based on a combination of clinical and biological characteristics, may help to provide a safer method for identification of patients potentially suitable for immunosuppressive drug weaning procedures.

Introduction

Organ transplantation is a treatment of choice for end stage diseases affecting vital organs. Its application has been progressively and successfully extended to new indications, particularly in aged patients. Furthermore, there is compelling evidence of continuous improvement in recipient and transplant survival over the last few decades that are attributed to not only a better control of the alloimmune response [1,2] but also a general improvement

in surgical techniques and clinical management, diagnostic tools and control of infectious neoplasia [3]. Side effects of immunosuppression, including infectious complications [4–6], malignancies [3,7] and metabolic disorders [8] contribute substantially to morbidity and mortality among transplant recipients [9]. Several reports [2,10–12] have repeatedly pointed towards unmodified rates of long-term graft loss, suggesting that most of these benefits are attributed to improvements in early patient management and particularly from a decrease in acute rejection (AR) incidence – the rate of which has indeed dropped to below 10% in the ''modern'' era. Recently, the more popular practice of systematic or ''protocol'' kidney graft biopsies has revealed an unexpectedly high incidence of early lesions attributable to calcineurin inhibitors (CNI), and histological lesions compatible with long-term calcineurin inhibitor exposure are observed in virtually all transplants in the long-term [10]. Thus ironically, long-term survival of kidney transplants, which initially benefited from modern immunosuppressive treatments, may now be principally limited by the effects of long-term exposure to drugs with direct nephrotoxicity and/or several other side effects [2,5,6,10]. In addition, the kidney toxicity associated with long-term CNI exposure is not restricted to permanent histological lesions but is also associated with functional modifications that are reversible upon CNI weaning [13], leaving room for a potential benefit of late CNI avoidance. These observations have progressively shifted the attention of clinicians towards a need for CNI minimization [14].

There is thus an urgent need to reassess the clinical status of long-term kidney transplant recipients for the rational design of immunosuppressive regimens. There is also a need to re-explore the scientific reasons underlying the stagnation of long-term survival despite improvements in immunosuppression, with particular focus on the current dramatic changes in donor and recipient demographics. It is therefore important to be able to more precisely understand how to assess rejection risk in patients who could be selected for safe immunosuppression minimization. Recently, it was suggested that the proportion of liver transplant recipients exhibiting operational tolerance and able to undergo successful and complete immunosuppressive withdrawal increased with time post-transplant (A. Sanchez-Fueyo, unpublished data) and represented a large population of patients at 10 years. There is currently a lack of such data in the field of kidney transplantation, particularly concerning the outcome of progressive immunosuppressive drug withdrawal 10 years after transplantation. Thus, one cannot formally exclude the possibility that a substantial proportion of long-term surviving kidney transplant recipients could be operationally tolerant, as in liver transplantation.

To identify biomarkers that may be used to select patients for safe immunosuppression withdrawal, we analyzed two clinically contrasted populations of kidney transplant recipients displaying either operational tolerance (defined as patients with stable graft function without any immunosuppressive treatment, who retain the capacity to respond to other immune challenges [15]), or chronic antibody-mediated rejection (CABMR). We hypothesized that the comparison of these two cohorts of patients would enable the identification of a gene expression profile that defines the ''minimal risk'' of rejection for a kidney transplant patients. In this paper, we analysed these biomarkers of operational tolerance on a cohort of 144 kidney transplant patients and we suggest that a rational approach to selection, based on a combination of both clinical and biological characteristics, may offer a more secure basis for CNI weaning [16].

Materials and methods

Patients and the Données Informatisées et VAlidées en Transplantation (DIVAT) data bank

Since 2003, 196 individuals were included in the study. The protocol was approved by the Ethical Committees of Nantes and Paris Universities. All patients signed an informed consent before inclusion. All patients were recruited within a collaborative project (GenHomme and PHRC grants, Research French ministry) involving the Nantes Institute of Transplantation, the Center for Adult Transplantation of the Necker Hospital (Paris, France) and the biotechnology company, TcLand Expression (Nantes, France). All of the patients had received a transplant between 1990 and 2005. The following inclusion criteria were applied: adult recipients who received a first kidney transplant from a deceased donor and who presented a stable graft function for at least 5 years with tacrolimus or cyclosporine A (CNI) for maintenance therapy associated or not with mycophenolate mofetil (MMF), azathioprine (AZA) and/or steroids. CNI trough levels were 75–250 ng/ml for cyclosporine and 5–15 ng/ml for tacrolimus. Patients were prescreened and designed as stable according to an estimated glomerular filtration rate above 40 ml/min, a stable creatinemia (±25% of the mean value of creatinemia in the year before the inclusion) and a daily proteinuria ≤1 g/day. These criteria had to be confirmed at 3 months after the enrollment of the patients in the study (period of inclusion) to confirm the stability of the patient. Of the 196 prescreened patients, 32 were not definitively included in the study because of a modification in their status between the 3-month period of inclusion. Twenty patients were excluded for serious adverse events (SAE) [cancer, post-transplant lymphoproliferative disorder (PTLD) or

serious systemic infection] that could modify their gene expression profile during the follow-up (SAE). Finally, 144 patients met these clinical criteria and were enrolled in the study. Blood samples collected on the day of enrollment were used to perform qPCR for the 49 genes of operational tolerance described in Reference [14]. The demographic characteristics of the patients are summarized in Tables 1 and 2. The demographic characteristics of the whole cohort of patients (n = 1870) transplanted in our center within the period 1990–2005 and presenting the same inclusion criteria as the 144 patients have been provided for comparison in Table 3a, b.

Histological analysis

A total of 33 patients (23%) showed a deterioration of their graft function after inclusion, during the follow-up. The 14 biopsies available for these patients were classified according to the updated Banff classification criteria as interstitial fibrosis and tubular atrophy (IF/TA), CNI toxicity or CABMR (defined by the diagnostic triad of circulating anti-donor specific antibodies with transplant glomerulopathy associated with IF/TA and/or peritubular capillary C4d deposition) [17]. The 19 remaining patients were not biopsied for technical reasons or medical contraindication.

Table 1. Description of the quantitative parameters for the 144 patients.

	Min	Mean	Max	SD
Age of the recipients (years)*	12.00	45.19	69	13.64
Age of the donor (years)*	8.00	36.61	69	14.70
Proteinuria (g/24 h)*	0.01	0.1925	1.01	0.165
Creatinemia (μM)*	68.00	119.20	202.00	30.24

*At inclusion.

Table 2. Description of the qualitative parameters for the 144 patients.

	Percentage	Number of patients
Male recipient	62.5	90
PRA*	13.9	20
HLA anti-class I*	4.2	6
HLA anti-class II*	7.6	11
DSA anti-class I*	0.0	0
DSA anti-class II*	5.6	8
Age of the recipient >55*	28.0	40 (1 NA)
HLA incompatibilities >4	20.8	30
Age of the donor >55	11.1	16
Male donor	72.2	104
ACEI/A2RA medication	41.0	59
Tacrolimus/CsA*	29/71	42/102
RIA*	70.4	100
Acute rejection episodes	13.8	20
Underlying kidney disease (glomerulonephritis)	16	23
Induction therapy (polyclonal Abs/monoclonal Abs/none)	39/30/31	57/44/43

ACEI, angiotensin converting enzyme inhibitor; CsA, cyclosporine A; HLA, human leukocyte antigen; PRA, panel reactive antibody; RIA, radio immuno assay.
*At inclusion.

Detection of allo-antibodies and CRP measurement

Human leukocyte antigen (HLA) antibodies were detected using a multiplex screening test (LAT-M; One lambda, Canoga Park, CA, USA). Donor-specific antibodies were detected using Luminex Single antigen (Labscreen Single Antigen; One lambda). CRP was measured on 100 μl serum samples stored at −80 °C.

Blood sampling, RNA extraction and cDNA preparation

Blood sampling was performed at the time of inclusion for the 144 patients using EDTA vacutainers. RNA was extracted from peripheral blood mononuclear cells using the TRIzol method (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. Genomic DNA was removed by DNase treatment (Roche, Indianapolis, IN, USA). RNA quality and quantity was determined using an Agilent 2100 BioAnalyzer (Palo Alto, CA, USA). RNA was reverse transcribed into cDNA using polydT oligonucleotide and Maloney leukemia virus reverse transcription (Invitrogen).

RNA cDNA and real time quantitative polymerase chain reaction (qPCR)

Real-time quantitative PCR was performed in an Applied Biosystems GenAmp 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). The gene signature of ''operational tolerance'' originally consisted of 49 genes identified using custom cDNA microarrays [16]. For the purpose of analyzing this signature using qPCR, primer and probe sets were manually re-designed to achieve the best correspondence between microarrays and qPCR. This resulted in nine genes having to be excluded because of poor efficacy, impossibility to design adequate probes, or lack of expression of the gene in question. As a result, optimization was successful for the following 40 genes: *AKR1C1*, *AKR1C2*, *AREG*, *AURKA*, *BTLA*, *BUB1B*, *C1S*, *CCL20*, *CDC2*, *CDH2*, *CHEK1*, *DEPDC1*, *ELF3*, *GAGE*, *HBB*, *IGFBP3*, *LTB4DH*, *MS4A1*, *MTHFD2*, *NCAPH*, *NR2F1*, *PARVG*, *PCP4*, *PLEKHC1*, *PLXNB1*, *PODXL*, *PPAP2C*, *RAB30*, *RASGRP1*, *RBM9*,

Table 3. Description of the quantitative (a) and qualitative (b) parameters for the 1870 patients transplanted in Nantes between 1990 and 2005.

(a) Quantitative parameters

	Min	Q1	Median	Mean	Q3	Max	NA	SD
Age of the recipients (years)*	18.00	36.00	47.00	46.46	57.00	80.00	35	13.25
Age of the donor (years)*	0.00	25.00	40.00	39.43	51.00	82.00	4	16.06

(b) Qualitative parameters

	Percentage	Number of patients	NA
Male recipient	63.5	1188	0
Immunization	50.3	576	726
Age of the recipient >55	28.1	515	35
HLA incompatibilities >4	22.3	415	8
Age of the donor >55	17.4	325	4
Male donor	69.0	1288	2
Tacrolimus	23.3	435	0

RGN, *RHOH*, *SLC29A1*, *SP5*, *SPON1*, *SYNGR3*, *TACC2*, *TLE4*, *TMTC3*, and *ZWILCH*. To quantify the levels of mRNA, we normalized the expression of the target genes to a set of five genes (B2M, HPRT, GAPDH, UBC, and YWHAZ) and one reference sample composed of a pool of RNA from 169 kidney transplant recipients. We employed the ΔΔCT method of relative quantification. Prior to any target gene measurement the presence of qPCR inhibitors was excluded by testing for *HPRT1* gene expression over three 5-fold dilutions.

Statistical analysis: predictor generation for operational tolerance

Based on the signature of 40 specific unique genes of operational tolerance that were validated using quantitative PCR [14], we identified the smallest list of genes able to correctly classify a training set composed of 14 blood samples equitably distributed between patients with operational tolerance [operationally tolerant patients (TOL)] and patients with chronic rejection [nonoperationally tolerant patients (N-TOL)] described in Reference [14] and randomly created using the PAM package [18] on the open-source statistical software ''R'' [19]. The validity of the prediction was tested using jacknife on genes and an independent test set of seven N-TOL and three TOL. Then, the validity of the prediction was tested using a resampling method with replacement ($n = 1000$), which means that a sample could be chosen more than once. The genes were selected and validated using the leave one out technique to obtain the lowest rate of N-TOL misclassification (higher sensitivity). This corresponds to the minimal value for which a sample will be classified as TOL. Using the training set, we identified 20 genes (Table 4) and a threshold of 0.96 that classified the training sets with the following performance criteria: 71.4% sensitivity, 100% specificity, 77.8% negative predictive value (NPV), and 100% positive predictive value (PPV) (Fig. 1). The area under the curve (AUC) of the receiver operating characteristic (ROC) curve for the training set was 0.92 (excellent discrimination). The predictive power of the 20 gene set was then tested on a test set composed of 10 patients (three TOL and seven N-TOL) and the following performance criteria were obtained: 100% sensitivity, 85.7% specificity, 100% NPV and 75% PPV. This predictor was then used to predict the TOL or N-TOL status of the cohort of 144 stable patients at a threshold of 0.96.

Table 4. Selection of the 20 genes of operational tolerance.

Genes of operational tolerance
RHOH
BUB1B
TMTC3
MS4A1
GAGE
C1S
RAB30
PLXNB1
AKR1C1
CCL20
NCAPH
AKR1C2
CDC2
SPON1
RGN
RBM9
DEPDC1
HBB
SYNGR3
CHEK1

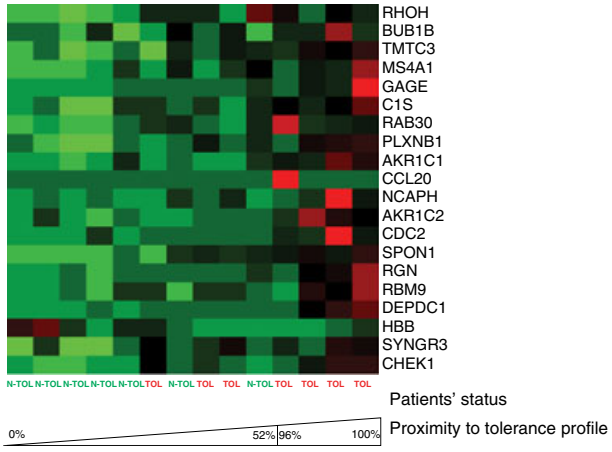

Figure 1 Expression of the 20 genes of operational tolerance in the training set. A threshold of 0.96 correctly classified the training set with the following performance criteria: 71.4% sensitivity, 100% specificity, 77.8% negative predictive value (NPV) and 100% positive predictive value (PPV).

Statistical analysis: probability of dysfunction according to the time since the 5th anniversary

The probability of dysfunction according to the time since the 5th anniversary of the transplantation have been performed on the 144 patients using an adaptation of the Kaplan and Meier estimator [20] to take into account the censoring and the truncation of the data (Fig. 2). The analysis of the relationships between each clinical factor and time until dysfunction was performed using the Cox model which was also extended to truncated data. Donor specific antibodies (DSA) were not analyzed because of too few patients with DSA positive antibodies (Table 5). To put things further into perspective, the data on the total number of patients with the same inclusion criteria [(transplanted within the period of 1990–2005 from deceased donor under tacrolimus or cyclosporine A (CsA)] have also been described (n = 1870).

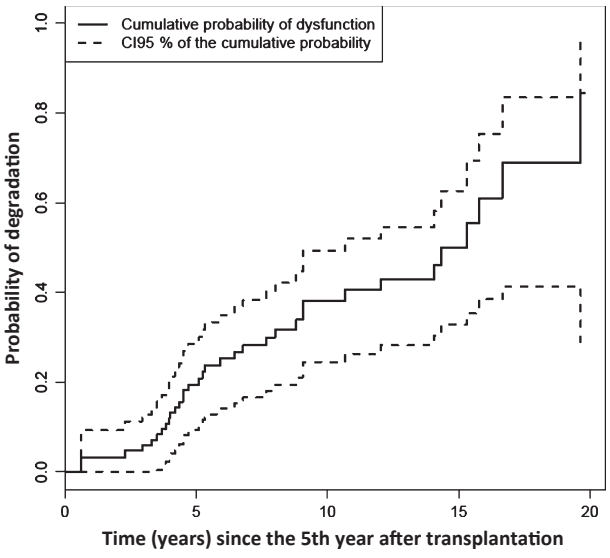

Figure 2 Probability of dysfunction of the 144 patients according to the time since the 5th anniversary of the transplantation.

Results

Clinically based patient selection enables the identification of a highly stable transplant population

Because long-term graft outcome may be principally limited by CNI side effects and that CNI minimization is becoming a major issue in kidney transplantation, we first assessed if patient selection based on simple clinical criteria could identify highly stable patients among long-term surviving recipients (≥5 years post-transplantation). All selection parameters were historical variables affecting long-term outcome. All 144 patients were tested at least 5 years post-transplantation with a median inclusion period of 2.5 (range 0.0–16.7) years. Among the population of 144 kidney recipients enrolled in the statistical analysis, all had received a first kidney transplant; 62.5% were male recipients. Mean recipient age was 45.2 (±13.6) years with 28% more than 55 years of age. Whereas all patients had a well-functioning kidney [estimated glomerular filtration rate (eGFR) ≥40 ml/min] with <1 g of daily proteinuria at inclusion, 33 patients (23%) showed a deterioration of graft function at a median of 7.5 years after inclusion (range 0.6–19.7). Mean creatinemia and proteinuria at inclusion were 119 ± 30.24 μM (min: 68, max: 202) and 0.19 ± 0.16 g/day (min: 0.01, max: 1.01) for the 144 patients. Among the 144 patients, 111 patients maintained stable graft function during the study period (mean creatinemia and proteinuria at inclusion was 117.8 ± 29.5 μM and 0.18 ± 0.15 g/day and 120.9 ± 32.8 μM and 0.26 ± 0.29 g/day at the last check-up) and were defined as "highly stable". Patient demographics and quantitative characteristics are presented in Tables 1 and 2.

Among the 33 patients (23%) who showed a deterioration of graft function during the study period, a transplant biopsy was performed in only 14 patients for whom there was no contra-indication or technical infeasibility. These biopsies were classified according to the updated Banff classification criteria [17] (Table 6). The majority of the patients (9/14) displayed lesions compatible with CNI toxicity. Two patients were identified as suspicious for CABMR (one with C4d deposition and one with class II donor-specific antibodies). All patients displayed concomitant lesions of IF/TA.

Table 5. Description of clinical parameters of the five patients displaying a profile of operational tolerance.

Variables	HR	CI 95% HR
Gender of the recipient (male versus female)	0.62	[0.31; 1.28]
Age of the recipient (>55 versus <55 years)	0.55	[0.22; 1.34]
HLA incompatibilities A + B + DR (>4 versus <4)	0.75	[0.29; 1.96]
Gender of the donor (male versus female)	0.93	[0.43; 2.03]
Age of the donor (>55 versus <55 years)	2.66	[1.01; 6.96]
RIA at inclusion	1.01	[0.64; 1.61]
Creatinemia at inclusion (μM)	1.00	[0.91; 1.44]
Proteinuria at inclusion (g/24 h)	1.61	[0.27; 9.71]
Anti-DSA at the time of the study	–	[–; –]
Anti-HLA (CI-I and CI-II) at the time of the study	0.98	[0.34; 2.90]
Induction therapy	–	[–; –]

RIA, radio immuno assay.

Table 6. Biopsies available for these patients were classified according to the updated Banff classification criteria.

Code	Diagnosis	Banff classification	C4d staining	DSA
GH023	CNI toxicity*	g2. i0. t0. v0. ptc2 cg1. ci3. ct3. cv1. ah3. mm0. ti3	Focal (<10%)	0
GH228	CNI toxicity*	g0. i0. t0. v0. ptc0cg0. ci1. ct1. cv1. ah2*. mm0. ti1	0	0
GH070	CNI toxicity (IgA nephropathy)*	g1. t0. i1. ah3. v0. cg0. ct1. ci1 mm0. cv0. ptc 1	0	0
GH083	CNI toxicity*	g2. i1. t0. v0. ptc1. cg0. ci1. ct1. cv0. ah3. mm0	NA	0
GH135	CNI toxicity*	g1. i0. t0. v0. ptc0. cg0. ci2. ct1-2. cv1. ah3. mm0	NA	0
GH134	CNI toxicity*	g0. i0. t0. v0. ptc0. cg0. ci2. ct2. cv2. ah3. mm0. ti0	0	0
GH154	CNI toxicity*	g0. i0. t0. v0. ptc0. cg0. ci1. ct1. cvx. ah3. mm2. ti1	0	0
GH096	CABMR*	g3. i1. t0. v?. ptc1. cg2. ci2-3. ct2-3. cv?. ah3. mm3. ti2	NA	Class II
GH053	CABMR (CNI toxicity)*	g3. i0. t0. v0. cpt0. cg3. ci1. ct1. cv2. ah3. mm2. ti0	Diffuse	0
GH168	Extra membranous glomerulonephritis*	cv2. v0. ah2. ptc0	0	0
GH063	IF/TA grade 2	gx. t0. i1. ah1. v0. cgx. ct2. ci2. mmx. cv0.	0	0
GH086	Nephroangiosclerosis*	g1. i0. t0. v0. ptc0. cg0. ci1. ct1. cv1. ah3. mm1. ti1	0	0
GH202	CNI toxicity*	g0. i0. t0. v0. ptc0. cg0. ci1. ct1. cv1. ah1-2. mm0		0
GH145	Mild fibrosis lesions*	g0. i1. t3. v0. ptc0. cg0. ci1. ct1. cv1. ah1. mm1. ti1	0	0

CNI, calcineurin inhibitor; IF/TA, interstitial fibrosis and tubular atrophy.
*With associated IF/TA.

Probability of dysfunction according to the time since the 5th anniversary of the transplantation and analysis of the relationships between each clinical factors and time until dysfunction

This analysis, in the limitation of the few number of patients we have, show that the patients whom we selected as ''highly stable'' are degrading their function in time and highly suggest that the stability of a patient at a given time does not preclude it survey in the future. Table 2 shows the relationships between each clinical factor and time until dysfunction (univariate Cox model). In the limit of the number of patients, the only significant risk factor associated with degradation is the age of the donor higher than 55 years (HR = 2.66, CI 95% = [1.01; 6.96]).

For comparison, the quantitative and qualitative pretransplant characteristics from the whole cohort of patients transplanted in our center between 1990 and 2005 were given on Table 3a and b. A total of 1870 patients under tacrolimus or CsA have been considered. The probability to have a functioning kidney 5 years after transplantation is 76% (95% CI = [76.5%; 80.3%]). Five years after their transplantation, 1347 alive patients are still observed with a functional kidney (taking into account the lost of follow-up). Fifteen years after transplantation, only 208 patients have still a functional graft (Fig. 3).

Blood transcriptional patterns associated with operational tolerance in highly stable selected patients

In a previous study, we identified several blood biomarkers, some of which were able to discriminate between operational tolerance and CABMR, such as the 49 genes associated with operational tolerance [16]. A statistical algorithm using a subset of these genes and generated as described in the Materials and Methods section was then

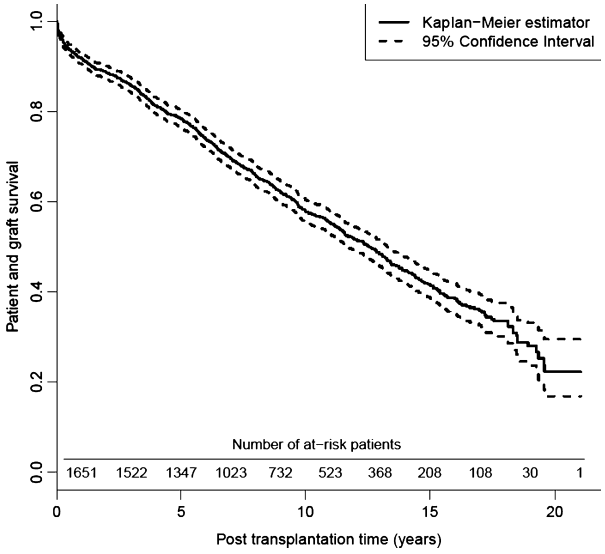

Figure 3 Patient and graft survival of the 1870 patients transplanted in Nantes between 1990 and 2005 according to the post-transplantation time.

used to diagnose the TOL/N-TOL status of the cohort of 144 highly stable patients. At a stringent threshold of 0.96, 5 of the 144 patients (3.5%) displayed the profile of operational tolerance according to the expression of the 20 most relevant genes for operational tolerance (Fig. 4). The proximity to the tolerance profile is indicated by a black bar. The dashed line indicates the threshold value above which a sample is classified as TOL (0.96). The clinical description and clinical follow-up of these patients are provided in Tables 7 and 8 and Fig. 4. Importantly, none of these patients displayed a deterioration of graft function.

Clinical case reports for the patients identified as "operationally tolerant"

Case 1 – GH211: L.M., born in 1957, whose initial disease was an interstitial nephropathy, received a deceased kidney transplant in 2000 from a 43-year-old donor with four HLA incompatibilities. She received no transfusion and presented low levels of panel reactive antibody (PRA, 3% class II) before transplantation. She received an induction therapy with Simulect® (Novartis Pharma, Bazel, Switzerland). Her immunosuppressive treatment consisted of MMF/corticosteroids (CS)/CsA until November 2005 and CS/CsA thereafter. She displayed no delayed graft function or AR episodes. During the first year of transplantation, she presented numerous serious bacteriological infections, a cytomegalovirus (CMV) disease and recurrent herpes. At 1 year post-transplantation her creatinemia was 107 μM and remained highly stable (107 μM) until inclusion in this study in 2004. At the time of writing, her renal function is stable without proteinuria (0.16 g/day).

Case 2 – GH182: J.M., born in 1934, presented a renal failure because of polycystic nephropathy. No pretransplant immunization was noted secondary to three blood transfusions. In 1999, she received a deceased kidney from a 69-year-old donor with six incompatibilities. After a short course of induction therapy with anti thymoglobulin (ATG), her maintenance regimen consisted of CS/MMF/CsA until 2007 and CsA/MMF thereafter. She developed delayed graft function. Her creatinemia at 1 year post-transplantation was 107 μM and remained stable (121 μM) and without proteinuria (0.09 g/day) until her enrollment in the study and throughout the study period.

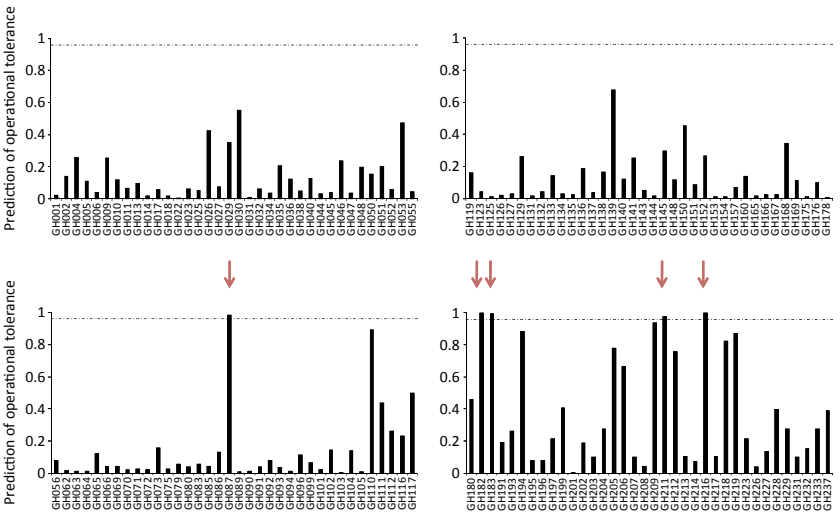

Figure 4 The statistical algorithm was used to predict the operationally tolerant patients/nonoperationally tolerant patients (TOL/N-TOL) status of the cohort of 144 highly stable patients. The proximity to the tolerance profile is indicated by the black bar. The dashed line indicates the threshold value above which a sample is classified as TOL (0.96).

Table 7. Analysis of the relationships between each clinical factors and time until dysfunction.

Case	Initial renal disease	Recipient age	Donor age	Time post-transplant (years)	Blood transfusion	Pregraft PRA	HLA incompatibility number	Induction therapy	Treatment	Delayed graft function	Acute rejection	Infection
Case 1 –GH211	Interstitial nephropathy	44	43	9	No	3% cl2	4	Simulect	CS/MMF/CsA and CS/CsA	No	No	Herpes CMV TUI
Case 2 – GH182	Polycystic nephropathy	68	69	11	3	0%	6	ATG	CS/MMF/CsA and CsA/MMF	Yes	Yes (1)	Zoster
Case 3 – GH087	IgA glomerulopathy	27	39	13	2	0%	6	No	MMF/CsA/CS	No	No	CMV acute pyelonephritis
Case 4 – GH216	IgA glomerulopathy	36	37	10	No	No	4	Simulect	CS/MMF/CsA and CsA/MMF	Yes	No	Prostatite herpes
Case 5 – GH183	IgA glomerulopathy	24	18	24	2	25% cl2	2	Simulect	CsA/AZA/CS and CsA/AZA	Yes	Yes (1)	No

AZA, azathioprine; CS, corticosteroid; CMV, cytomegalovirus; CsA, cyclosporine A; MMF, mycophenolate mofetil.

Table 8. Functional follow-up of the five patients with a profile of operational tolerance.

	Creatinemia	Proteinuria	DSA
Case 1 – GH211			
1 year before	107	0.33	0
Inclusion	107	0.16	0
1 year	104	0.11	0
2 years	106	0.13	0
3 years	114	0.1	0
4 years	109	0.14	0
Case 2 – GH182			
1 year before	107	0.11	0
Inclusion	121	0.09	0
1 year	134	0.25	0
2 years	134	0.1	0
3 years	150	0.1	0
4 years	150	0.06	0
5 years	125	0.21	0
Case 3 – GH087			
1 year before	106	0.07	0
Inclusion	97	0.14	0
1 year	114	0.13	0
2 years	96	0.13	0
3 years	95	0.35	0
4 years	93	0.13	0
5 years	102	0.44	0
6 years	101	0	0
Case 4 – GH216			
1 year before	144	0.24	0
Inclusion	130	0.19	0
1 year	143	0.26	0
4 years	127	0.1	0
Case 5 – GH183			
1 year before	174	0.1	0
Inclusion	187	0.11	0
1 year	197	0.27	0
3 years	212	0.1	0
4 years	217	0.12	0

Case 3 – GH087: H.D., born in 1970, developed an IgA glomerulopathy. He received two transfusions before transplantation but no HLA immunization was detected. In 1997, he received a renal transplant from a 39-year-old deceased donor with six incompatibilities. He did not receive an induction therapy and his maintenance immunosuppressive regimen consisted of MMF/CS and CsA. He displayed no delayed graft function and had no AR episodes. He presented a primo-infection with CMV the month following transplantation as well as an episode of acute pyelonephritis. At 1 year post-transplantation his creatinemia was 106 μM and in 2005 (at inclusion), it was 97 μM, with no proteinuria (0.07 g/day).

Case 4 – GH216: C.C., born in 1964, developed an IgA glomerulopathy. He did not receive any transfusions before transplantation and no HLA immunization was

detected. In 2000, he received a kidney from a 37-year-old deceased donor with four HLA incompatibilities. He received an induction therapy with Simulect® and his maintenance therapy consisted of CS, MMF and CsA for 2 months and then CsA/MMF. He experienced a delayed graft function. During the first year of transplantation, he presented numerous bacteriological and virological infections. Ten years after transplantation, his renal function was good with a creatinemia of 144 μM 1 year before inclusion, which remained highly stable at inclusion (130 μM) and throughout the study period, with no proteinuria.

Case 5 – GH183: J.T., born in 1963, developed an IgA glomerulopathy. He was transfused twice before transplantation at which time 25% Class II PRA was detected. In 1986, he received a renal transplant from a 24-year-old deceased donor with two HLA-A-B-DR incompatibilities. He received an induction therapy with Simulect® and his maintenance immunosuppressive regimen consisted of CsA/AZA/CS and then CsA/AZA. He suffered from a delayed graft function and presented one AR episode 24 months after transplantation. After 10 years of transplantation (1999), he had a creatinemia of 211 μM (eGFR 53 ml/min) and his proteinuria was 0.12 g/24 h. At 1 year post-transplantation his creatinemia was 174 μM and in 2005 (at inclusion) it was 187 μM, with 0.11 g/day proteinuria.

Note that he experienced a slow and progressive decline of his renal function. This could be probably explained by the aging of his kidney rather than to a chronic immunological activity according to the lack of concomitant DSA or to a recurrence of his initial disease (i.e. no proteinuria). A polyomavirus nephropathy could not be excluded despite its lower probability under CSA for maintenance therapy. However, in the absence of histological proof this diagnosis could not be definitively excluded.

To better characterize these five patients, we also looked at other immunological and inflammatory markers such as DSA and CRP. HLA antibodies and Donor-specific antibodies were measured at the time of inclusion and were all found negative.

Three patients displayed a normal CRP levels [GH211 (case 1) 3.0 mg/l, GH182 (case 2) ≤3 mg/l, GH216 (case 4) ≤3 mg/l] and two patients displayed slightly elevated CRP [GH087 (case 3) 14.5 mg/l and GH183 (case 5) 14.4 mg/l] without any concomitant signs of infection or other patent clinical explanation.

Discussion

Kidney transplantation remains the best treatment for end-stage renal diseases. During the past decade, the incidence of AR has dramatically decreased. Thus, the current major problems post-transplantation include chronic rejection, BK virus infection (probably linked to the increased immunosuppressive power of recent immunosuppressive drugs such as tacrolimus and MMF), and side effects of long-term immunosuppressive drug intake [3,4,7,8]. One goal of nephrologists is thus to define patient eligibility for immunosuppressive drug weaning, which could improve graft survival and patient quality of life in the long-term. Histological examination of graft biopsies is the gold standard for assessing recipient status [17] but this invasive procedure cannot be easily repeated in stable patients. There is thus a need for reliable, predictive, noninvasive and repeatable tools to identify stable recipients with a low-graft rejection risk i.e. biomarkers. Biomarkers aim at the measurement and evaluation of ''normal'' or pathogenic biological processes in response to therapeutic intervention [21]. These should be quantitative, early, predictive and noninvasive. Genetic, transcriptional and proteomic tools can all be employed to identify potential biomarkers that could be useful to define patient eligibility for CNI interruption or minimization procedures.

In this study, all patients were recipients of a first transplant and all selected parameters were known variables affecting long-term outcome. The inclusion criteria were based on a well and stable functioning kidney (>40 ml/min eGFR) at inclusion, without significant proteinuria. Altogether, these data can be used to select a population of transplant patients more than 5 years post-transplantation with a stable kidney graft function over time between their transplantation and their inclusion in the study. Nevertheless, 33 of these patients experienced a deterioration of their graft function in the long-term after inclusion. The majority of the biopsies available from these patients presented lesions compatible with CNI toxicity and only two patients presented lesions of CABMR and/or lesions of IF/TA. These data suggest that selection based on clinical parameters is an absolute necessity to engage patients in a secure immunosuppression weaning protocol. However, such selection does not guarantee safety and has to be completed at least by a graft biopsy and checking for the presence of anti-HLA antibodies, which was not scheduled in the time-frame of our study in 2002 where such analyses were not routinely performed. This reinforces the need for biological biomarkers as surrogate markers of immunological risk before embarking on weaning procedures. Tolerance is the ultimate goal of physicians and researchers working in the field of transplantation. An impressive body of studies has been carried out in animals where long-term graft tolerance against MHC mismatched combinations is commonly achievable [22]. However, immune tolerance fulfilling the same stringent criteria has not yet been

obtained in nonhuman primates or in the clinic [23]. Detecting such a tolerant state and associated biomarkers is thus another key issue that needs to be resolved. Such markers should not only be able to identify a state of tolerance in patients under immunosuppressive therapy, but should also be able to predict future loss of graft function before the first clinical symptoms appear.

Given that some patients continue to have well-functioning grafts after immunosuppression withdrawal, one could speculate that a certain percentage of renal transplant patients under immunosuppression are susceptible to becoming spontaneously tolerant after immunosuppression weaning. The development of diagnostic tests could open up the possibility of rationally designed immunosuppressive-weaning protocols. Accumulation of favorable genetic predisposing factors are also feasible but have not yet been explored. We have identified a small biomarker panel using gene-expression profiling of peripheral blood from spontaneously tolerant renal transplant recipients [16].

In this previous study, performed on 75 renal transplant patients including 17 patients with operational tolerance, a biological footprint of 49 genes was identified by microarray technology [16] that was further reduced to a 40-gene set that provided robust class prediction by qPCR. Interestingly, this footprint was also identified in one of 12 patients with stable graft function under standard triple immunosuppression and five of 10 patients on low-dose steroid monotherapy. This not only suggests that these patients are potentially operationally tolerant and could benefit from immunosuppression weaning but also that the signature of operational tolerance may be found in patients under immunosuppression, which is a major prerequisite for initiation of weaning. This was reinforced by the loss of the peripheral signature in some patients that correlated with a change in clinical phenotype from operational tolerance to rejection [16].

This paper first shows that our cohort of ''highly selected'' patients on clinical parameters degrade function in time. The dysfunction probability curves of the 144 patients suggest that a clinical selection on usual parameters (such as proteinuria and estimated graft function) is not sufficient to predict graft outcome. In the limit of the number of patients studied, these data also confirm that usual risk factor well established at the first times of the transplantation are not so clear for patients still alive with a functioning kidney at 5 years (except for the donor age) [24–26].

Thanks to the use of advanced molecular techniques, we then identified five patients that displayed the profile of operational tolerance among a cohort of patients with stable kidney graft function. First, this analysis confirms that it is possible to identify a tolerance-like profile under immunosuppression as indicated previously [16], suggesting that some biological investigations may be possible in large cohorts of patients with stable graft function under immunosuppression. Surprisingly, these patients only represent 3.5% of the selected stable patient population and 4.6% of the highly stable patients, who remained stable from the time of transplantation until now, which is extremely low compared with tolerant liver recipients. Indeed, some data [14,27] report that a high proportion of liver recipients exhibit operational tolerance following immunosuppression withdrawal and that this phenomenon dramatically increases with time (~6% at 3 years, 33% at 6 years and >60% at 10 years post-transplantation) (A. Sanchez-Fueyo, unpublished data). Thus, this first study strongly suggests that this phenomenon is less frequent in kidney transplant recipients, even a long time after transplantation (at least 5 years). Of course, this report is only speculative as there is actually a lack of data concerning the effect of a progressive immunosuppressive drug withdrawal years following kidney transplantation. Nevertheless, some elements may account for this difference. First, we previously reported that operational tolerance to a kidney transplant was ''metastable'' and a full state of tolerance was not achieved in all patients [28]. Whereas some ''operationally tolerant'' patients presented a humoral response following influenza vaccination similar to that of healthy volunteers, others had a poor response, comparable with that of the immunosuppressed recipients [28]. Second, the peripheral blood gene expression profile of liver recipients with drug-free long-term graft function [14] is very different from that observed in kidney recipients [16]. As an example, gene expression analysis revealed enrichment in genes encoding for a variety of NK cell-surface receptors, $CD8^+$ and $\gamma\delta TCR^+$ T cells in blood from tolerant liver recipients whereas the signature is mainly related to B cells in operationally tolerant kidney recipients [29–31]. We also have to take organ specific differences into account. A previous study in a murine transgenic model revealed different organ susceptibilities to rejection with kidney grafts evoking stronger T-cell proliferation and differentiation than in other organs [32]. Finally, note that one patient (GH183, case 5) was identified as a tolerant-like patient despite the fact that he presented one AR episode 24 months after transplantation. This observation is concordant with our previous report where we showed that among the 10 cases of operationally tolerant recipients, half had suffered from AR episodes in their ''transplant life'' [28], showing that AR is not incompatible with a future functional profile of operational tolerance.

We also looked at other immunological markers such as DSA, HLA mismatch and CRP at the time of inclusion or in the 3 months following inclusion. The five selected

patients were all DSA negative. No significant difference was found for HLA mismatch. CRP was measured on serum from these five patients that display normal or low CRP levels (cases 3 and 5) suggesting low-grade inflammation without the indication of an inflammatory event at the time of analysis. Interestingly, four of the five patients who display a profile of operational tolerance also harbors TRIB-1 value compatible with a profile of ''high stability'' [33] (data not shown).

Of course, such a study does not provide formal proof that operational tolerance can be achieved in these patients and the fact that only 3.5% of these patients are really tolerant is also a hypothesis that only a weaning/minimization of immunosuppression could confirm. The literature is now ''chock-a-block'' with potentially interesting biomarkers that need to be tested on large cohorts of patients. The next step is now to validate these biomarkers and enter patients into immunosuppression weaning procedures on a large scale. Given the ultimate deterioration of some patients within this population, this study teaches us that limiting the selection to single clinical parameters is not enough to carefully perform such CNI weaning procedures and probably has to be associated with the absence of immunological signs of activity such as circulating anti-HLA antibodies and histological lesions of antibody-mediated rejection. This is the objective of several teams within several European and American networks [30,31].

Authorship

MGi, JPS and SB: designed research. ALB, MG, AD, ED, LL, AP, CGG and GE: performed research. MG, ACG, ET and CL: contributed new reagents or analytic tools. MG, JPS, ALB and SB: analyzed data. SB: wrote the paper. YF: performed statistical analysis.

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