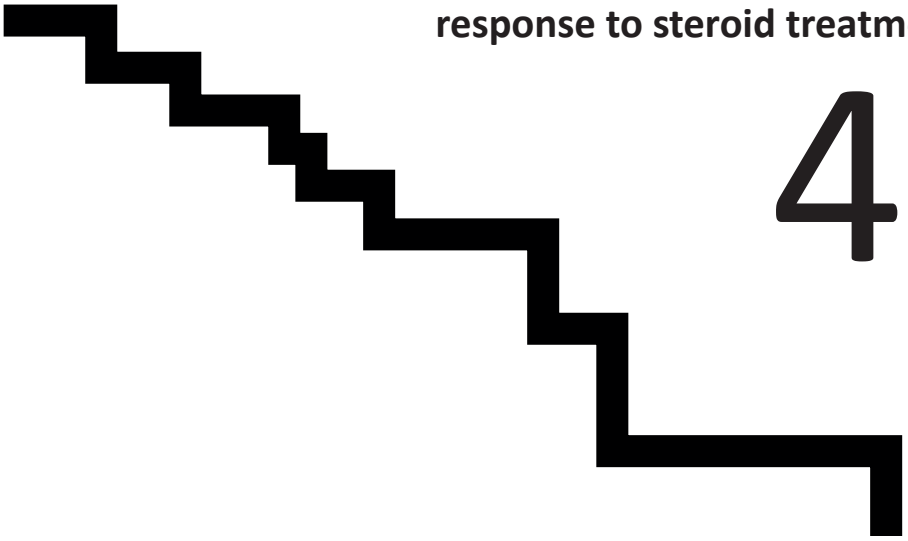
Molecular and genetic markers for the prediction of kidney transplant outcome

Yang, J.

Endothelial-epithelial-related transcriptional levels in acute rejection biopsies of kidney transplant recipients are predictive for a worse response to steroid treatment



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Jianxin Yang, Marijke Spruyt-Gerritse, Jacqueline Anholts,
Frans H.J. Claas, Michael Eikmans*

Department of Immunohematology and Blood Transfusion,
Leiden University Medical Center, Leiden, the Netherlands.

Abstract

Background: Molecular assessment of transplant biopsies may help in prognostic assessment. We investigated whether transcriptional alterations in acute rejection biopsies provide information on outcome.

Method: We studied a cohort of patients transplanted between 1995 and 2005. Eighty-five biopsies taken due to clinical acute rejection (median 14 days [9.5-35.0] post-transplant) were analyzed for 23 T-cell mediated rejection (TCMR)-related transcripts (including CD28, lag-3, CD8a, granulysin, ICOS, BTLA) and 13 endothelium-epithelium-related transcripts (including PECAM1, ICAM2, von Willebrand factor, E-selectin, CD34, caveolin 1) using the Fluidigm high throughput RT-PCR system.

Results: TCMR transcripts as well as the endothelium-epithelium-related transcripts clustered together in principal component analysis. Therefore, for each patient a composite score, resulting in a T-score and E-score, respectively, was calculated. Five control transplant biopsies, containing no morphologic abnormalities, showed a profile of high E-score and low T-score.. In the low E-score group with rejection (n=46), 52.2% of the patients showed resistance to steroid treatment, whereas in the high E-score group (n=39) this was 28.2% ($P<0.05$). Both the T-score and rejection severity according to Banff criteria were not significantly associated with response to steroid treatment.

Conclusion: A molecular signature of relatively low transcripts levels of endothelium-epithelium-related genes may reflect injury of the microvasculature in the allografts, posing a risk factor for decreased therapy response. The results suggest that molecular assessment of the graft tissue has an added value to histomorphologic evaluation.

Introduction

Acute allograft rejection remains a risk factor for adverse transplant outcome (1). Rejection is associated with infiltration of host inflammatory cells and allograft injury. Histologic assessment according to the Banff classification is used to determine the type of rejection, but may be of limited prognostic value.

Gene expression assessment in the biopsy tissue may represent an objective means of analysis and a complementary tool to conventional diagnostic measurements. Numerous studies have described molecular markers in blood, urine, and graft tissue, which are associated with acute rejection (2-7). Prediction of the therapeutic response to steroid treatment remains difficult using only clinical and histomorphologic parameters. Sarwal and colleagues reported that patients with steroid resistant rejection display elevated expression of T cell, natural killer cell, and B cells (3). Subsequent studies did not show an association of B cell infiltrates with steroid resistant rejection and graft function (8-10). The extent of staining of C4d, CD68, HLA-DR, and granulysin, and an elevated expression of Fas ligand in the graft tissue were associated with steroid resistance rejection (2). Rekers and colleagues showed increased expression of metallothioneins and LAG-3 and CD25:CD3e ratio in biopsy samples associated with steroid resistant rejection (11, 12).

Microvascular injury is associated with rejection: preservation of microvascular integrity and absence of inflammation are important for maintaining long term graft function (13, 14). Nickeleit et al reported that endarteritis, defined as the presence of inflammatory cell in the sub-endothelial space and adherence of mononuclear cell to endothelial cells, is associated with steroid resistant rejection (15). Haas et al confirmed that especially type 2B rejection (severe intimal arteritis comprising >25% of the luminal area) leads to a worse response to steroid therapy (16). Ozdemir et al showed that destruction of the microvasculature, as reflected by loss of endothelial markers, is associated with steroid resistant rejection (17).

In the current study, we examined 23 TCMR and 13 endothelium-epithelium related transcripts using the Fluidigm high throughput RT-PCR system in acute rejection renal biopsies, and investigated their relation to clinical outcome.

Materials and methods

Patient characteristics

Patients who underwent kidney transplantation at the Leiden University Medical Center (LUMC) between 1995 and 2005 were investigated. Biopsy samples were taken within 6 months after transplantation from 85 patients with clinical suspicion of acute rejection and 5 patients without histological rejection. Immunofluorescent C4d staining on 80

biopsy samples were performed as described previously (8). Donor specific antibody (DSA) information was not routinely available in this study. Biopsy samples were assessed blindly by two pathologists according to Banff 2011 criteria (11, 18). Informed consent was obtained from all individuals. Patient characteristics are shown in Table 1.

Selection of genes

Genes included for expression profiling were selected from previous studies. The endothelium-epithelium-related transcripts were selected based on probes that were differentially expressed between endothelial cells and non-endothelial cells (19), and between biopsies with ABMR and other biopsies (20, 21): CDH13, PLA1A, ROBO4, TM4SF18, GNG11, PGM5, KLF4, CAV1, CDH5, vWF, CD34, PECAM1, MCAM. The set of TCMR related molecules contained transcripts that were increased highest in expression in T cell mediated rejection biopsies compared to other transplant biopsies (22, 23): SLAMF8, TNFSF8, CD96, SIRPG, BTLA, SLA, ANKRD22, CD28, CD274, SP140, SH2D1A, ADAMDEC1, IL12RB1, LAG3, PTPN7, CD72, CD8a, CXCL13, CXCL10, GNLY, ICOS, RARRES3, TOX2. Majority of the primer sets were designed to target separate exons, spanning at least one intron (> 800 bp) to prevent amplification of genomic DNA.

RNA extraction and cDNA synthesis

RNA isolation and quality check were performed as described previously (12). Total RNA (50-200 ng) was used for cDNA synthesis, following the manufacturer's manuals.

High throughput qPCR analysis using Fluidigm 96.96 dynamic array

Ten times diluted cDNA (1.25 uL) was pre-amplified containing 2.5 uL of Taqman Preamp master mix (Applied Biosystems, Texas, USA) and 1.25 uL of pooled primer mix for 14 cycles. The qPCR reactions were performed using Eva-green dye following the Fluidigm protocol, and results were collected on the BioMark HD system. Absolute Cq values from duplicate measurements of each transcript were averaged, and relative gene expression levels were normalized to the geometric mean of the reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin.

Statistical analyses

Relative expression level were log2 transformed and normalized using the z-score for each transcript. The normalized z-score were averaged for each distinct set of transcripts and for each patient. Frequency of patients with steroid resistant rejection in high and low score groups were analyzed by chi-square test. Difference in score of individual endothelial related transcript between steroid responsive and resistant rejection were analyzed using T-test. Death-censored graft survival curves were created using the Kaplan-Meier method, and differences between curves were calculated using log rank tests.

Results

Demographics and clinical data

We studies 85 biopsy samples from patients with a clinical indication of acute rejection and 5 protocol biopsies without any indication of rejection. Acute rejection were treated by intravenous methylprednisolone. Thirty five patients had a steroid-resistant rejection, and fifty had a steroid responsive rejection. Steroid resistance was defined as no response to steroid treatment and requirement for antithymocyte globulin therapy within 14 days after the start of the steroid treatment, as described previously (11, 12). Clinical parameters and histologic lesions were not associated with steroid response treatment (Table S1).

Table 1. Demographics of patient cohort.

Variable	Number (%)
Recipient age (>= 50 year)	37 (43.5%)
Recipient gender (Female)	27 (31.8%)
Donor age (>= 50 year)	34 (40%)
Donor gender (Female)	55 (64.7%)
Donor type (Living)	20 (23.5%)
Time from transplant to rejection (days, IQR)	14 (9.5-35)
First transplantation (Yes)	71 (83.5%)
HLA AB-matching (Yes)	12 (14.1%)
HLA DR-matching (Yes)	25 (29.4%)
DGF in deceased donor (Yes)	23 (35.4%)
Steroid responsiveness	50 (58.8%)
Cold ischemia time (<= 18 h)	19 (22.4%)
Induction therapy (IL-2R blocker)	26 (30.6%)
Maintenance therapy	
Corticosteroid, CNI	34 (40%)
Corticosteroid, MMF	4 (4.7%)
Corticosteroid, CNI, MMF	34 (40%)
Banff score	
Glomerulitis (g=0/1/2/3)	56/20/3/2
Interstitial inflammation (i=1/2/3)	36/27/18
Tubulitis (t=0/1/2/3)	3/29/32/17
Intimal arteritis (v=0/1/2/3)	44/20/4/6
Interstitial fibrosis (ci=0/1/2)	51/24/6
Tubular atrophy (ct=0/1/2)	47/32/2
C4d staining (diffuse positive)	11(13.8%)
Graft survival (Death censored)	
>1 year	81 (95.3%)
>6 year	79 (92.9%)

Eleven (13.8%) biopsies showed diffuse C4d positive staining. The median (IQR) time of biopsy was 14 (9.5-35) days post-transplant. Twenty-six (30.6%) patients received IL-2 receptor blocker monoclonal antibody as induction therapy. Thirty-eight patients (44.7%) received a double drug regime (corticosteroid, calcineurin inhibitor or MMF) and thirty-four patients (40%) received a triple drug regime (corticosteroid, calcineurin inhibitor, and mycophenolate mofetil) as maintenance immunosuppressive therapy (Table 1).

HLA, human leukocyte antigen; DGF, delayed graft function; IQR, Interquartile ranges; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil.

Steroid resistant rejection predicts inferior long term graft outcome

The effect of steroid resistant rejection on long term graft survival was assessed. Patients showing a poor response to steroid treatment had inferior long term graft survival (66.4%) compared to patients with a steroid-responsive rejection (95.4%, P=0.022, Figure 1).

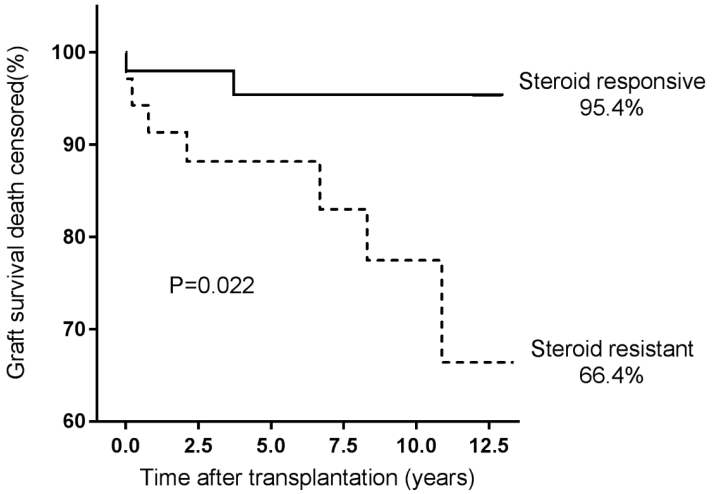

Figure 1. Association of steroid resistant with long term graft survival. Steroid resistant rejection were significantly related with inferior graft survival after kidney transplant.

Fluidigm dynamic array produce consistent result with conventional qPCR.

RNA from the transplant biopsies with acute rejection was subjected to gene expression assessment by the Fluidigm dynamic array system. To validate this system, two transcripts were additionally tested using conventional qPCR. The absolute Cq values highly correlated between Fluidigm dynamic array and conventional qPCR (R^2>0.92), indicating that the Fluidigm dynamic array generated comparable results with conventional real time PCR (Figure S1).

Transcripts were clustered into two groups

The normalized gene expression data were analyzed based on principal component analysis (PCA). Both the endothelial-epithelial related transcripts (E-group) and TCMR related transcripts (T-group) clustered together by the first component of PCA (Figure 2). Based on this observation, we decided to calculate for each patient the composite score of the endothelial-epithelial related transcripts (termed as E-score) and of the TCMR related group transcripts (termed as T-score).

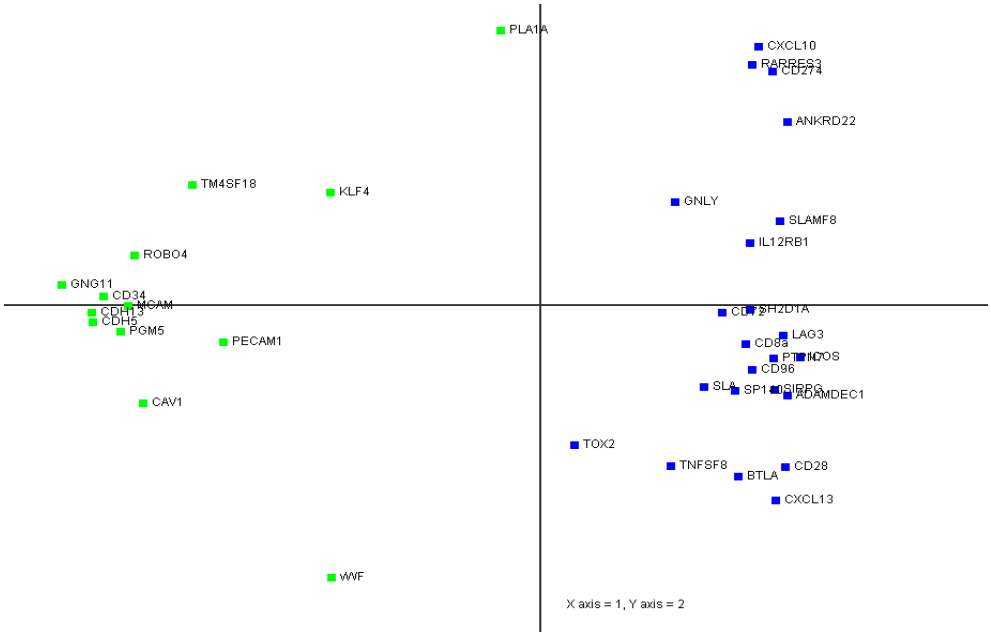

Figure 2. Principal component analysis for clustering the transcripts. The first component could divide the transcripts into two groups except the E-selectin: Endothelial-epithelial related group (green dots) and T cell mediated rejection related group (blue dots).

E-score associated with steroid resistant rejection

The patient group with acute rejection was divided into two groups based on the E-score: E-high (E-score>0, n=39) and E-low (E-score<0, n=46). In the E-low group 52.2% of the patients showed resistance to steroid treatment, which was significantly higher than the 28.2% of patients in the E-high group (P=0.025, Figure 3 and Table 2). The E-score was not associated with any of the Banff lesions (Table S2).

Table 2. Relationship between molecular scores and steroid response treatment.

	Steroid responsive (N=50)	Steroid resistant (N=35)	P
E-score			0.025*
E-score<0	22 (47.2%)	24 (52.2%)	
E-score>0	28 (71.8%)	11 (28.2%)	
T-score			
T-score<0	18 (62.1%)	11 (37.9%)	0.662
T-score>0	32 (57.1%)	24 (42.9%)	

*P values were calculated using Chi-Square test.

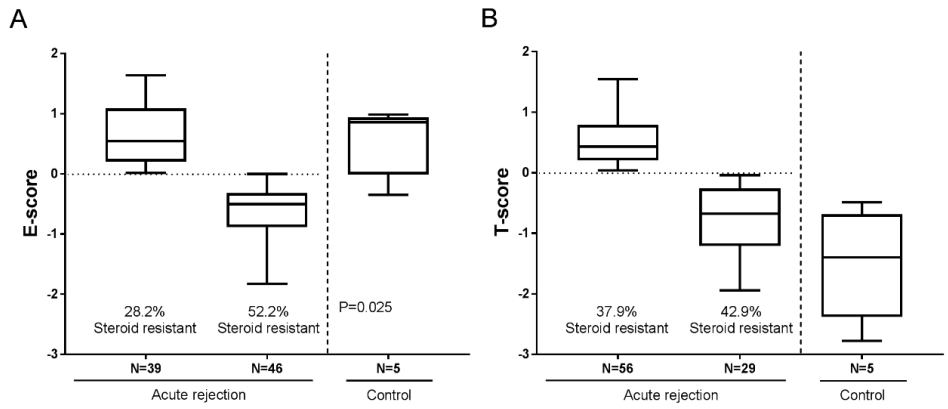

Figure 3. Molecular score associated with steroid resistant rejection. In the E-low group 52.2% of the patients showed resistance to steroid treatment, which was significantly higher than the 28.2% of patients in the E-high group. The T-high and T-low group were not significantly different in the percentage of patients having steroid resistant rejection

The association of individual endothelial related transcript with steroid resistant rejection was analyzed. Patients with steroid resistant rejection showed significantly lower expression of TM4SF18, PGM5, and CD34 compared to patients with steroid response treatment after multiple correction (Table 3).

The patients without rejection showed relatively high E-score and low T-score (Figure 3).

T-score not associated with steroid resistant rejection but was related to Banff inflammation score

The patient group with acute rejection was divided into two groups based on the T-score: T-high (T-score>0, n=56) and T-low (T-score<0, n=29). The T-high and T-low group were not significantly different in the percentage of patients having steroid resistant rejection (37.9% versus 42.9%, Figure 3 and Table 2).

The T-score was significantly correlated with interstitial inflammation and tubulitis (Table S2). The frequency of patients with i>1 was 71.2% in the T-high group, and 27.6% in the T-low group (P<0.005). Similarly, 82.7% of patients in the T-high group had moderate or severe tubulitis (t>1) in contrast to 20.7% in the T-low group (P<0.0001). The T-high group also had significantly higher incidence of interstitial fibrosis (46.1%) than the T-low group (26.7%, P=0.048). The T-score was not associated with intimal arteritis.

Table 3. Association between individual endothelial-epithelial related transcripts and steroid response treatment.

	Steroid responsive (N=50)	Steroid resistant (N=35)	P
CAV1	0.231 ± 0.796	-0.345 ± 1.204	0.017
CD34	0.204 ± 0.856	-0.500 ± 0.972	0.001*
CDH13	0.157 ± 0.893	-0.344 ± 1.093	0.023
CDH5	0.146 ± 0.938	-0.340 ± 1.018	0.026
GNG11	0.189 ± 0.956	-0.417 ± 0.900	0.004
KLF4	0.233 ± 1.119	-0.289 ± 0.746	0.018
MCAM	0.137 ± 0.909	-0.282 ± 1.098	0.058
PECAM1	0.234 ± 0.911	-0.341 ± 1.046	0.008
PGM5	0.193 ± 0.923	-0.447 ± 0.925	0.002*
PLA1A	0.131 ± 0.925	-0.213 ± 1.105	0.122
ROBO4	0.171 ± 0.933	-0.404 ± 0.969	0.007
TM4SF18	0.236 ± 0.897	-0.473 ± 0.968	0.001*
vWF	0.173 ± 0.809	-0.100 ± 1.040	0.178

[a] Gene expression data shown as mean ± SD.

*P values were calculated by T-test and adjusted by Bonferroni method (P<0.00385).

Discussion

In the present study mRNA expression levels of endothelium-epithelium related transcripts (E-score) and T-cell mediated rejection related transcripts (T-score) were investigated in transplant biopsies at time of acute rejection (AR). We found that a relatively low E-score is associated with resistance to steroid treatment, whereas the T-score and Banff score were not related to outcome of the rejection. The T-score is significantly associated with interstitial inflammation and tubulitis. Results from this study suggest that molecular assessment offer an added value to histologic diagnosis with respect to predicting of steroid therapy.

Evaluation of multiple markers that belong to a similar pathophysiologic pathway is superior to tests of single markers, as it may decrease the variation introduced by aberrant expression and by inter-laboratory differences. The Fluidigm dynamic arrays system provides a high throughput gene expression platform on the basis of real time quantitative PCR, which requires very low input amounts of nucleic acid. Our data, consistent with other studies, show that the microfluidic technology has high concordance with conventional qPCR (24, 25). The automated microfluidic chip system enables faster analysis, and it significantly reduces the reagent and sample consumption (26).

T-group transcripts, previously described to be elevated in TCMR biopsy samples compared to all other conditions (23), mainly reflect T cell co-stimulation, activation and signalling, and cytotoxic T cell- and INF-γ-related effects. Majority of the biopsies obtained in the previous study were taken more than one year after transplantation (6). We interrogated these transcripts in a cohort of biopsies, containing acute rejection, most of which had been taken within 3 months after transplantation). Consistent with a previous study (22), the relative high T-score was significantly associated with the extent of interstitial inflammation and tubulitis, but not with intimal arteritis. Thus, assessment of T-score in biopsy samples provides additional value on diagnosis of TCMR.

Here we found that decreased expression of endothelium and epithelium related transcripts is associated with resistance to steroid pulse treatment in kidney transplantation. In contrast, Sis et al reported that endothelial associated transcriptional levels were elevated in late biopsies diagnosed with ABMR (21). Sellares et al subsequently demonstrated that molecular transcripts that typified biopsies with ABMR were mainly expressed in endothelial, epithelial cells, and NK cells, which was confirmed by another prospective study (20, 27). The seemingly contradicting clinical effect of dysregulated expression of endothelial cell transcripts between previous studies and ours may be explained by a difference in the time period of the biopsies after transplantation (early versus late), and the type of the rejections studied. Our cohort mostly contained TCMRs on the basis of histomorphology. Unfortunately, we cannot completely rule out a humoral component for the minority of cases that showed C4d positivity, since serum for donor specific antibody screening at time of rejection was not always available.

Endothelial cells in glomeruli and peritubular capillaries mediate critical processes such as inflammation and coagulation. Decreased expression of endothelium and epithelium related transcripts, which are involved in blood vessel development and biological adhesion, may reflect low nephron integrity and reduced nephron repair after injury. The analysis of individual transcripts showed that TM4SF18, PGM5, and CD34 remain significantly lower in patients with steroid resistant rejection after Bonferroni correction. TM4SF18 is a member of transmembrane 4 large six family, characterized by four conserved transmembrane domains (28). The tetraspanins can form a large protein complex with integrin and growth receptors, which may mediate cell adhesion, proliferation, and migration (29, 30). PGM5 is involved in cell-to-cell adherens junctions of endothelial and epithelial cells (31-33). CD34 is mainly expressed on endothelium, epithelium, and human hematopoietic stem cells, and it is a potential marker of endothelial progenitor cells (34). In combination with MCAM, PECAM1, CAV1, ROBO4, PGM5, and cadherin molecules, these molecules are involved in angiogenesis during wound healing and in cell adhesion, all in the context of enhanced repair capacity and kidney integrity. Clinically this may mean that the renal function is more likely to recover after high-dose steroid therapy when the kidney has a higher repair capacity and nephron integrity, as reflected by the relatively high expression of the endothelial transcripts

studied. This notion is supported by the observation that severe vascular destruction is related to worse response to steroid treatment, compared to mild vascular destruction (17). Furthermore, severe intimal arteritis and adherence of mononuclear cells to endothelial cells were associated with steroid resistant rejection (15, 16).

In conclusion, we found that decreased expression of endothelial-epithelial transcripts in the biopsy during acute rejection, as reflected in a low E-score, is significantly associated with a poor response to steroid treatment. The expression of TCMR-related transcripts, as reflected in the T-score, and Banff lesions in the biopsy were not associated with steroid response. Molecular assessment of biopsies at moment of rejection may provide additional support for clinical diagnosis. The prognostic value of the expression profiles studied for predicting steroid resistant rejection would need to further tested in a prospective study.

Acknowledgments

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Supplementary Data

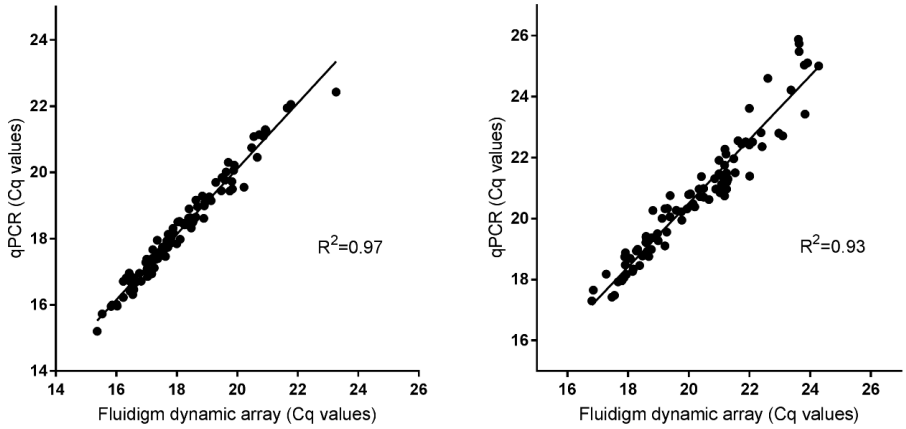

Figure S1. Absolute Cq values of Fluidigm dynamic array and conventional real time PCR. The highly correlated results suggested comparable results between Fluidigm technique and conventional qPCR .

Table S1. Clinical parameters and histologic lesions were not associated with steroid resistant rejection

	Steroid responsive (N=50)	Steroid resistant (N=35)	P
Recipient age (years, IQR)	48.5 (36.75-53)	47 (37-55)	0.96
Donor age (years, IQR)	47 (32.5-56.5)	46 (36-59)	0.58
Donor type			0.15
Living	9	11	
Cadaveric	41	24	
First transplantation [a]			0.72
Yes	42	29	
No	7	6	
HLA-ABDR matching [a]			1
Full Matching	5	4	
Mismatching	44	31	
DGF in deceased donor			0.42
Yes	16	7	
No	25	17	
Glomerulitis (g)[b]			0.37
0	35	21	
1	11	9	
2	2	1	
3	0	2	
Interstitial inflammation (i)[b]			0.12
1	20	16	
2	20	7	
3	8	10	
Tubulitis (t)[b]			0.39
0	2	1	
1	18	11	
2	21	11	
3	7	10	
Interstitial fibrosis (ci)[b]			0.52
0	30	21	
1	13	11	
2	5	1	
Tubular atrophy (ct)[b]			0.96
0	28	19	
1	19	13	
2	1	1	
Intimal arteritis (v)[c]			0.46
0	30	14	
1	10	10	
2	2	2	
3	3	3	

[a, b, c] data missing for one, four, eleven patient

*P values were calculated using Chi-Square test or Fisher's Exact Test.

Table S2. Relationship between group score and histologic lesions

	E-score<0 (N=43)	E-score>0 (N=38)	P	T-score<0 (N=29)	T-score>0 (N=52)	P
Glomerulitis (g)			0.88			0.13
0	31	25		16	40	
1	10	10		11	9	
2	1	2		1	2	
3	1	1		1	1	
Interstitial inflammation (i)			0.60			5.84E-4*
1	17	19		21	15	
2	15	12		6	21	
3	11	7		2	16	
Tubulitis (t)			0.54			5.88E-8*
0	1	2		2	1	
1	13	16		21	8	
2	18	14		6	26	
3	11	6		0	17	
Interstitial fibrosis (ci)			0.43			0.048*
0	29	22		23	28	
1	10	14		4	20	
2	4	2		2	4	
Tubular atrophy (ct)			0.29			0.75
0	27	20		18	29	
1	16	16		10	22	
2	0	2		1	1	
Intimal arteritis (v) [a]			0.52			0.92
0	25	19		14	30	
1	9	11		7	13	
2	3	1		2	2	
3	2	4		2	4	

[a]data missing for seven patients

E-score, Endothelial-epithelial group score; T-score, TCMR related group score

P values were calculated using Chi-Square test or Fisher's Exact Test.