Original Paper

Molecular Patterns of Subclinical and Clinical Rejection of Kidney Allograft: Quantity Matters

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Key Words

Kidney transplantation • PCR • Protocol biopsy • Subclinical rejection • Transcriptome • Prostaglandin receptor

Abstract

Background/Aims: Subclinical rejection diagnosed from protocol biopsies is thought to be a risk factor of long-term allograft dysfunction. The reason why in some patients subclinical rejection does not represent risk for progression is not fully understood. **Methods:** The intragraft expression of 376 target genes involved in chemokine defense, apoptosis, inflammation, tolerance and TGF- β signalling pathways was measured using quantitative real-time RT-PCR ($2^{-\Delta\Delta C_t}$) method in subclinical inflammation (SCI, n=10), clinical inflammation in acute T-cell mediated rejection (CI, n=10) and no rejection samples (n=9). **Results:** Clinical inflammation group showed an increased expression of genes for chemotaxis mediating cytokines (*CCL1*, *CCL17*, *CCL24*, *CCL25*, *CCL26*), cytokine receptors (*CCR1*, *CCR2*, *IL1RAPL2*, *CXCR5*), proinflammatory cytokines (*IL12A*, *LTA*), inflammatory mediator (*PTAFR*), complement protein C3, executioner protein of apoptosis (*CASP7*), growth factor (*TGFA*), colony stimulating factor (*CSF-2*), proteins involved in dendritic cells differentiation and interaction (*CD209*, *LAMP3*), regulation of immune response (*LILRB2*, *LILRB4*). The quantitative difference in transcripts signature between SCI and CI is consistent with stronger proinflammatory setting of CI. Prostaglandin E2 receptor gene expression was independently associated with lower risk of further graft function deterioration (OR 0.11, CI 0.01-0.78, $p < 0.0001$). **Conclusion:** Subclinical acute kidney inflammation has transcriptional profile of immune injury of lower extent compared to clinical acute inflammation.

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Introduction

Despite improvements in first year of kidney allograft survival, chronic rejection remains to be the main reason for late kidney allograft loss [1]. In the development of chronic rejection, a subclinical alloimmune injury may play an important role [2]. The incidence of subclinical acute rejection may vary and depends on time post transplantation, immunosuppressive regimen and previous sensitization [1, 3-6]. Therapy of subclinical rejection remains to be a subject of debate. While some authors referred benefits of subclinical rejection treatment [7-10], others did not [11, 12].

The explanation why subclinical rejection is not accompanied by functional deterioration in some patients remains to be speculative [9, 11, 13]. A different phenotype and cytokine activity of infiltrating cells [14] along with quantitative difference in transcriptional activity have been suggested to be responsible for the absence of graft function deterioration [15].

Although subclinical rejection of kidney allograft might have negative impact on allograft survival and some investigators have attempted to account for differences between SCI and clinical acute rejection (CI) on molecular level [16-19], no detailed differences have yet been fully elucidated. Transcriptome alterations detected by molecular analysis might even precede minor subclinical infiltrates in histology of protocol biopsies [20]. Several attempts have been made to predict short- and long-term graft outcome by molecular analyses of protocol biopsies [21, 22]. The identification of such molecular predictors and thereby grafts at risk seems to be crucial for future biomarker-driven therapy in transplantation.

We hypothesized that molecular phenotype evaluation might provide novel insight into the pathogenesis and clinical relevance of subclinical rejection of kidney allograft. Thus we evaluated the molecular phenotype of subclinical inflammation (SCI) from 3-month protocol biopsies and clinical inflammation (CI) from case biopsies and searched for potential molecular predictors of graft function deterioration.

Patients and Methods

Study Design and Patients

To analyze molecular phenotype of distinct clinical course of subclinical and clinical rejection, we identified study patients among kidney transplant recipients from deceased donor transplanted in 06/2005-06/2008 whose part of biopsy specimens was archived for transcriptome analyses. Rigorous sample selection was based on morphologic finding according to Banff 2005 classification. Patients with BK nephropathy have been excluded from the study.

Subclinical inflammation group (SCI) (n=10) involved patients with finding of borderline changes or acute T-cell mediated rejection (TCMR) in protocol biopsy at month 3 in the absence of clinical dysfunction (increase in S-Cr < 10%, no proteinuria) and with no history of prior rejection [9]. Clinical inflammation group (CI) (n=10) involved kidney recipients with borderline changes or acute T-cell mediated rejection in early case biopsy within 3 months after kidney transplantation which was performed due to clinical signs of graft function deterioration. Control group (n=9) consisted of patients with normal histological findings in protocol biopsies performed in stable renal allografts at 3 months after transplantation. Acute rejection was treated according to KDIGO clinical practice guidelines including treatment of borderline changes and subclinical rejection [23]. All patients were followed for 24 months. The Ethics Committee of the Institute for Clinical and Experimental Medicine approved the study protocol and all patients signed informed consent to participate in the study (IRB approval number G08-08-10). This study was conducted in accordance with Helsinki Declaration.

Immunosuppressive Treatment

Immunosuppression consisted of tacrolimus (TAC), mycophenolate mofetil (MMF), steroids in all patients but two from CI group who received maintenance therapy based on cyclosporine A. These two patients did not experience graft function deterioration. Two patients (1 SCI, 1 control group) were given induction treatment with basiliximab and one with rabbit antithymocytary globulin (SCI). As one of the major factors that may influence the development of SCI is suboptimal immunosuppression we carefully

looked for adequacy of immunosuppressive treatment which was adequate and comparable across the studied groups.

Protocol Biopsy

Protocol kidney graft biopsy was performed in accordance with our centre practice at 3 months after transplantation; early case biopsy was performed within 3 months postTx. Biopsies were obtained under ultrasound guidance (Toshiba, Power Vision 6000) using a 14-gauge Tru-Cut needle (Somatex®; Medical Technologies GmbH, Germany). A small portion (2mm) of the cortical zone of the biopsy specimen was immediately placed in the RNA later Stabilization Reagent (Qiagen) and stored at -20 °C or -80°C until RNA extraction.

RNA Extraction and cDNA Synthesis

The entire procedure, ranging from RNA extraction to cDNA synthesis, is in detail described elsewhere [24].

Real-time RT-PCR Analysis Using TaqMan Low Density Arrays (TLDA)

Gene expression profiling was performed using a custom-made Taqman® low density array (TLDA, Applied Biosystems, CA, USA) to analyze 376 candidate genes known to have implications in immune response (chemokine defence, apoptosis, inflammation, tolerance and TGFβ signaling). Quantitative real-time RT-PCR assay based on TLDA technology was performed as described elsewhere [25]. Real-time RT-qPCR data were quantified using the SDS 2.4 software package (Applied Biosystems, Foster City, CA, USA).

Expression Data Analysis

Relative gene expression values were generated from TLDA analysis using the comparative $2^{-\Delta\Delta Ct}$ method for relative quantification, which is implemented in the Applied Biosystems Relative Quantification (RQ) Manager Software v1.2.1 (Applied Biosystems, Foster City, CA, USA) [26]. For calculating the RQ of target genes in transplanted kidney, renal tissue from protocol biopsy with normal histological finding, not included in control group, was designated as calibrator. Using the $2^{-\Delta\Delta Ct}$ method, data are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the calibrator sample. All qRT-PCR data were rigorously inspected before included into analysis. All evaluated samples expressed more than 60% of target genes.

Immunohistochemistry

Immunohistochemical detection of prostaglandin E receptor 2 /PTGER 2/ was performed on 4 µm-thick paraffin sections of early case and 3 month protocol renal graft biopsies using a two-step indirect method. The slides were deparaffinized in xylene and rehydrated in graded ethanol. Heat-induced epitope retrieval in the buffer was used to improve the reactivity of antibody. Endogenous peroxidase was blocked by 0.3% H₂O₂ in 70% methanol for 30 minutes. Primary antibody was applied (PTGER 2, origin: rabbit, clone: polyclonal, Acris, dilution: 100x, application: 30 min RT, buffer: citrate, pH 6). Detection of antibody was performed using Simple Stain MAX PO (MULTI) Universal Immuno-peroxidase Polymer, Anti-Mouse and -Rabbit -Histofine (Nichirei, Japan). Finally, specimens were stained with Dako AEC + High Sensitivity (Dako, Glostrup, Denmark) for 10 min and were counterstained with Mayer's hematoxylin.

Statistical Analysis

Student *t* test and the analysis of variance were applied to the variables following a normal distribution. Based on gene expression data distribution, we performed nonparametric testing and data were expressed as median and interquartile range. Group comparison for median RQ values was determined using Kruskal-Wallis test. Post-hoc-inter-group comparisons were made using Mann-Whitney test with adjustment for multiple comparisons. Significance was defined as a two sided *p* value < 0.05. Association between continuous variables (RQ gene expressions, allograft function) and ordinal variables (Banff scores) was assessed as Spearman correlation. The genes considered to be differentially expressed had not to fulfil only statistical but also biological significance, when minimal cut-off for differential expression was set to 2-fold. Multivariate logistic regression analysis was conducted to search for significant associations of possible predicting factors with respect to the outcome variable (progression of renal dysfunction). Due to low patient number, logistic regression modelling was restricted to 5 significant genes from univariate analysis of the highest biological and statistical relevance. The area under the ROC curve (c-statistic) derived from this

Table 1. Patients' demographics and clinical characteristics according to studied groups

	control group (n=9)	SCI (n=10)	CI (n=10)	P
Recipient age (yr)	45 ± 13.26	52.8 ± 5.63	54.7 ± 10.586	0.183
Recipient gender (% male)	5 (55.6)	5 (50)	9 (90)	0.128
Mean % PRA at transplantation	6.22 ± 10.32	15.2 ± 31.74	15 ± 31.34	0.721
Previous transplant	0	0	0	1
HLA mismatch	3.89 ± 1.05	2.9 ± 0.99	4 ± 0.82	0.031
Cold ischemia time (hr)	17.61 ± 2.86	17.44 ± 4.09	17.04 ± 2.06	0.942
Dialysis vintage (d)	669 (307-1330)	642 (422-1474)	462 (314-813)	0.535
Grafts with delayed graft function (%)	2 (22.2%)	5 (50%)	1 (10%)	0.123
Graft function development (d)	1.67 ± 3.39	4.6 ± 4.93	0.9 ± 2.85	0.088
Biopsy (postTx d)	99 ± 9.55	94.5 ± 4.17	28.7 ± 31.41	0.001
Donor age (yr)	41.56 ± 12.27	44.3 ± 15.84	46.43 ± 12.88	0.899
Maintenance immunosuppressive regimen at biopsy				NS
Tacrolimus, MMF, Steroids	9 (100%)	10 (100%)	8 (80%)	
Cyclosporine A, MMF, Steroids	0	0	2 (20%)	
Serum creatinine (μmol/L)				
Baseline (at biopsy)	113 ± 28.95	133.7 ± 40.19	244 ± 96.61	0.0003
yr 1	114.56 ± 33.00	233.97 ± 214.3	211.33 ± 147.17	0.229
yr 2	150.66 ± 108.68	225.42 ± 199.23	227.08 ± 140.82	0.491
GFR estimated with MDRD (ml/sec/1.73m ²)				
Baseline (at biopsy)	1.05 ± 0.22	0.78 ± 0.27	0.49 ± 0.15	<0.0001
yr 1	1.06 ± 0.34	0.66 ± 0.36	0.61 ± 0.30	0.12
yr 2	0.89 ± 0.29	0.64 ± 0.38	0.55 ± 0.26	0.069
Proteinuria (g/d)				
Baseline (at biopsy)	0.13 ± 0.12	0.38 ± 0.46	0.67 ± 0.48	0.24
yr 1	0.066 ± 0.073	0.787 ± 0.779	0.254 ± 0.342	0.011
yr 2	0.194 ± 0.156	0.821 ± 1.304	0.319 ± 0.357	0.22

Data are expressed as mean ± SD or median and interquartile range.

logistic regression analysis reflects the model's discriminative power. Unsupervised hierarchical clustering that grouped together genes with similar expression patterns was performed with MultiExperiment Viewer 4.6.0 (TM4, Boston, MA, USA).

Results

Patients' Characteristics and Outcome

In total, 29 renal allograft biopsies were studied. Out of these samples, ten samples were derived from protocol biopsies with subclinical rejection including borderline changes (SCI), ten samples from early case biopsies with finding of clinical rejection and borderline changes (CI). Nine patients with normal histological finding in protocol biopsies served as control group. Transplantation-associated risk factors such as cold ischemia time, PRA level, etc. were not significantly different between studied groups (Table 1) except for total HLA mismatch and the day of biopsy taken resulting from the study setting. Among the patients studied, 8 patients developed delayed graft function (DGF) defined as the need for dialysis during the first week after transplantation [27], with 5 of these patients being from SCI group.

Histological Evaluation of Studied Biopsies

Severity of rejection grades was comparable across rejection groups. No difference in inflammation rate was observed between SCI and CI group. No active antibody mediated rejection was included into our study. The histological lesions scores show comparable chronic graft injury across the studied groups (Table 2).

Differential gene expression in biopsies derived from SCI and CI

To detect subtle alterations associated with the absence of graft function deterioration in SCI, mRNA expression profile of kidney allografts was compared. This analysis identified 23

Table 2. Histological evaluation of kidney biopsies according to Banff 05 classification

	SCI (n = 10)	CI (n = 10)	control (n = 9)	p- Value [†] (SCI vs. CI)	p-Value*
borderline/gradeI/gradeII	(4/3/3)	(3/2/5)			
i- score	1 (0-2)	2 (0.75-2)	0 (0-0)	0.162	0.001
t- score	1 (0-2)	1 (0.5-2)	0 (0-0)	0.76	0.002
ci- score	0 (0-1)	0 (0-0.25)	0 (0-0)	0.342	0.105
ct- score	1 (0-1)	1 (0.75-1)	1 (0-1)	0.423	0.440
v- score	0 (0-1)	0 (0-0.25)	0 (0-0)	0.473	0.093
cv- score	1 (0.5-2)	1 (0-1)	0 (0-1)	0.140	0.113
g- score	0 (0-0)	0 (0-0.25)	0 (0-0)	0.146	0.139
cg- score	0 (0-0)	0 (0-0)	0 (0-0)	1	1
mm- score	0 (0-0)	0 (0-0)	0 (0-0)	0.317	0.401
ah- score	0 (0-1)	1 (0-1)	1 (0-1.5)	0.426	0.548

Dara are expressed as median (interquartile range).

† according to the Mann- Whitney U test, no corrected for multiple comparisons

* according to Kruskal Wallis test

Table 3. The genes showing different expressions between SCI and CI group

target gene	SCI	CI	control	P # (CI vs. SCI)	n-fold (CI vs. SCI)	P *
C3	1.61 (0-6.04)	11.65 (2.16-49.63)	2.02 (0-2.76)	0.015	7.24	.021
CASP7	0.47 (0.22-0.88)	1.27 (0.89-2.3)	1.13 (0.59-1.17)	0.006	2.70	.014
CCL17	0.92 (0.26-3.87)	10.38 (0.89-48.69)	0.16 (0-0.71)	0.034	11.28	.004
CCL1	0 (0-0)	3.05 (0-9.08)	0 (0-0)	0.031	N/A	.017
CCL24	0 (0-0)	2.4 (0.57-6.42)	0 (0-0)	0.013	N/A	.004
CCL25	1 (1-1)	13.41 (1-75.06)	1 (1-1)	0.007	13.41	.001
CCL26	0.28 (0-1.98)	3.51 (0.84-27.89)	0.07 (0-1.02)	0.012	12.54	.009
CCR1	0.52 (0-1.35)	2.02 (0.59-3.52)	0 (0-0)	0.049	3.88	.017
CCRL2	0.61 (0-1.84)	3.33 (1.09-7.74)	0.5 (0-1.99)	0.018	5.46	.036
CD209	0.25 (0-0.7)	1.02 (0.51-3.09)	0 (0-0)	0.032	4.08	.001
CSF2	0.39 (0-0.09)	7.83 (1.08-47.03)	0 (0-0)	0.040	20.08	.002
CXCR5	0.02 (0-0.8)	0.37 (0.15-4.25)	0 (0-0)	0.049	18.50	.005
FOXP3	0.07 (0-0.77)	0.78 (0.19-4.1)	0 (0-0)	0.033	11.14	.013
HPGD	0.83 (0.32-1.27)	0.2 (0.12-0.48)	0.9 (0.71-2.13)	0.013	4.15	.001
IL12A	0 (0-0)	0.84 (0.54-2.82)	0 (0-0)	0.004	N/A	.020
IL1RAPL2	1 (1-1)	9.24 (1-50.55)	1 (1-1)	0.039	9.24	.004
KNG1	1.43 (0.79-2.5)	0.49 (0.1-0.87)	2.06 (1.43-3.77)	0.002	2.92	.001
LAMP3	0 (0-0)	3.67 (0.89-9.01)	0 (0-0)	0.008	N/A	.003
LILRB2	0.65 (0-4.96)	9.49 (1.37-20.17)	1 (0.09-1.95)	0.045	14.60	.049
LILRB4	0.36 (0.15-1.1)	2.12 (0.45-6.73)	0.16 (0-0.44)	0.034	5.89	.008
LTA	0.01 (0-0.2)	0.45 (0.12-1.24)	0 (0-0)	0.024	45.00	.027
PTAFR	1.11 (0.48-1.92)	4.04 (1.06-10.5)	0.4 (0.1-1.42)	0.041	3.64	.021
TGFA	0.35 (0-1.13)	1.18 (0.89-3.47)	0 (0-0)	0.028	3.37	.033

Only differentially expressed genes fulfilling statistical and biological significance criteria are shown (# according to Mann Whitney U-test). For complex view, results of Kruskal Wallis test* for all studied groups are shown.

genes with different expression between SCI and CI (Table 3). CI group showed a significant upregulation of genes for chemotaxis mediating cytokines (*CCL1*, *CCL17*, *CCL24*, *CCL25*, *CCL26*), cytokine receptors (*CCR1*, *CCRL2*, *IL1RAPL2*, *CXCR5*), proinflammatory cytokines (*IL12A*, *LTA*), inflammatory mediator (*PTAFR*), complement protein C3, executioner protein of apoptosis (*CASP7*), growth factor (*TGFA*), colony stimulating factor (*CSF-2*), proteins involved in dendritic cells differentiation and interaction (*CD209*, *LAMP3*) and in regulation of immune response (*LILRB2*, *LILRB4*) compared to SCI. We observed not only quantitative difference between two rejection groups, but also trend of increasing expression of proinflammatory genes rising from control through subclinical to clinical acute rejection group.

Furthermore, we were able to detect higher expression of Treg-specific cell marker, 15-hydroxyprostaglandin dehydrogenase (*HPGD*) and kinogen-1 (*KNG1*) ($p < 0.01$) in control and subclinical samples compared to clinical inflammation. An additional gene of interest was *FoxP3*, regulator of the development and function of regulatory T cells [28], which

showed lower gene expression in SCI biopsies compared to CI ($p < 0.01$).

Associations of molecular phenotype with histological scores for inflammation

In order to determine whether different gene expression profile of subclinical and clinical acute rejection was not related to severe grades of cellular rejection in CI, we correlated RQ of significant genes with the histological inflammation scores. This analysis revealed mostly positive correlation of some of evaluated genes with Banff score for tubulitis and interstitial inflammation but no correlation with glomerulitis, intimal arteritis was found (data not shown). We neither found association of these 23 differently expressed genes with DGF, future graft rejection nor function deterioration within 2 years posttransplantation (data not shown).

Unsupervised hierarchical clustering

Unsupervised hierarchical cluster analysis applied to all dataset did not produce a dendrogram with well-defined cluster groups. Performing unsupervised hierarchical clustering restricted to 23 differentially expressed genes revealed a significant up-regulation of proinflammatory genes in clinical inflammation group. Furthermore, it correctly classified all 10 CI samples to separated cluster (Figure 1). Out of four SCI samples clustered together with CI samples, two belonged to patients with consequent graft function deterioration and failed within two years and one belonged to patient with subclinical finding of severe TCMR grade II.

Graft function deterioration

Despite notably worse baseline renal function in CI group, no differences between SCI and CI were found at the end of year 2 (Table 1). However, some patients showed renal function deterioration during the follow-up. Four patients from SCI group and three patients from CI group experienced further renal function worsening caused by repeated rejection episodes. Additionally, one patient from control group experienced an episode of clinical acute TCMR within the first year (day 186) due to noncompliance. Hereinafter, he experienced renal function worsening due to repeated urinary tract infections and lost his graft due to abscessed pyelonephritis 25 months after kidney transplantation. The demographics and clinical characteristics of progressors and nonprogressors are summarized in Table 4.

Patient and graft survival

In total, two patients from SCI and one patient from CI group lost their graft due to repetitious rejection episodes (month 10, 11, 4, respectively). No graft loss due to patient death was observed.

Fig. 1. Unsupervised hierarchical clustering analysis using 23 differentially expressed genes. Higher gene expression of selected genes was observed in CI samples (shown in red colour). Unsupervised hierarchical clustering analysis demonstrated a different gene expression pattern for clinical inflammation samples by their separation from the others.

Table 4. Patients' demographics and clinical characteristics according to the groups based on graft function deterioration

	Nonprogressors (n = 13)	Progressors (n = 7)	P
Recipient age (yr)	54.7 ± 9.6	52 ± 5.4	0.504
Recipient gender (% male)	10 (76.9)	3 (42.9)	0.174
Mean % PRA at transplantation	14.3 ± 28.5	16.6 ± 36.9	0.88
Previous transplant	0	0	0
HLA mismatch	3.54 ± 0.88	3.29 ± 1.38	0.621
Cold ischemia time (hr)	16.15 ± 1.57	17.86 ± 4.88	0.400
Dialysis vintage (d)	606.8 ± 331.3	999 ± 856.9	0.155
Delayed graft function (%)	2 (15.38)	3 (42.85)	0.290
Graft function development (d)	2.08 ± 4.01	4.00 ± 5.00	0.384
Biopsy (postTx d)	49.69 ± 43.43	83.71 ± 21.55	0.087
Donor age (yr)	53.92 ± 6.37	42.71 ± 19.99	0.194
Maintenance immunosuppressive regimens at biopsy (%)			0.521
Tacrolimus, MMF, Steroids	11 (84.61)	7 (100%)	
Cyclosporine A, MMF, Steroids	2 (15.39)	0	
Serum creatinine (μmol/L)			
Baseline (at biopsy)	202.1 ± 87.7	164.3 ± 100.6	0.154
yr 2	148.7 ± 29.0	370.2 ± 224.5	0.057
ΔCr (yr 2—mo 3)	- 53.3 ± 81.2	205.9 ± 209.4	0.0004

Data are expressed as mean ± SD or n (%).

Differential expression of selected candidate genes is indicative of graft function deterioration

Next, we investigated whether evaluated transcriptome showed different phenotype in allografts suffering from later graft function deterioration. 7 patients that experienced worsening of graft function during 2 year follow-up were marked as progressors. Patients from control group were not involved into analysis when looking for potential predictors of renal function deterioration as all patients except for the aforementioned noncompliant one have stable renal function and because of different pre- and post- biopsy clinical course free of rejection and antirejection treatment.

Biopsy samples of progressors and nonprogressors were compared in order to identify genes of possible predictive value. 55 genes discriminating between progressors and nonprogressors were identified (Table 5). All genes fulfilled biological and statistical selection criteria. To evaluate whether these candidate genes are indicative of graft function deterioration, we performed multiple logistic regression analysis including five most biologically and statistically relevant genes (BNIP3L, PTGER2, CD1B, NFKBIE, MAPK 14) known from the univariate analysis to be differentially expressed in progressors. Multiple logistic regression analysis revealed that prostaglandin E2 receptor (PTGER2) gene expression level was associated with lower risk of graft function deterioration in studied biopsies (OR 0.11, 95% CI 0.01 – 0.78, Nagelkerke R² = 0.743, p < 0.0001). The receiver operator characteristic analysis confirmed low PTGER2 gene expression to be associated with progression of renal dysfunction (p < 0.0001, AUC 0.878, Figure 2).

The role of PTGER2 in further graft function deterioration was also confirmed at the protein level. Immunohistochemical (IHC) evaluation of kidney graft biopsy specimens showed higher intensity of PTGER2 staining in tubular epithelial cells in patients without progression of graft dysfunction within the follow-up (Figure 3).

Discussion

The explanation why subclinical inflammation is not accompanied by graft function deterioration varies. This study attempted to find possible explanation for silent course of

Table 5. The genes showing different expressions between progressors and nonprogressors

	Nonprogressors	Progressors	n-fold	P
BNIP3L	1.50 (1.04-1.92)	0.76 (0.69-0.96)	2.0	0.002
PTGER2	3.70 (1.71-15.16)	0.59 (0-1.37)	6.3	0.002
CD1B	6.15 (1.14-12.65)	0.09 (0-1.09)	72.4	0.004
MAPK14	1.44 (0.98-2.16)	0.57 (0-0.89)	2.5	0.006
NFKBIE	1.44 (0.52-3.51)	0.29 (0-0.56)	4.9	0.006
CCL17	7.18 (1.06-38.29)	0.30 (0.14-1.1)	23.8	0.008
IL1R1	1.34 (0.84-5.13)	0.45 (0.18-0.61)	3.0	0.008
NFKB1	2.64 (1.46-4.21)	0.91 (0.82-1.16)	2.9	0.008
PLA2G4C	1.26 (0.86-2.59)	0.47 (0.31-0.95)	2.7	0.008
LTB4R	2.71 (0.76-5.36)	0.68 (0.35-0.98)	4.0	0.01
CXCL6	13.45 (2.24-40.23)	1.16 (0.68-5.73)	11.6	0.013
SOC5	2.88 (1.13-5.2)	0.70 (0.53-1.14)	4.1	0.013
CD1C	2.11 (0.55-4.46)	0.21 (0.16-0.5)	9.9	0.016
HRH1	1.85 (0.57-5.62)	0.37 (0.35-1.13)	5.0	0.016
IL2	1.21 (0.05-4.01)	0.00 (0-0.19)	12110.0	0.016
SELP	1.23 (0.55-1.71)	0.22 (0.1-0.54)	5.7	0.016
CCL19	3.08 (0.33-8.6)	0.15 (0.1-1.38)	21.1	0.019
CCL26	2.39 (0.48-24.16)	0.13 (0-0.91)	18.1	0.019
SPARC	1.29 (0.74-1.49)	0.56 (0.52-0.79)	2.3	0.019
FCGR2B	2.72 (0.73-12.04)	0.68 (0.18-0.87)	4.0	0.024
IL10	4.74 (0.73-12.78)	0.00 (0-2.47)	47400.0	0.026
CD209	0.86 (0.58-2.46)	0.00 (0-0.91)	8605.0	0.027
CD28	2.48 (0.51-7.01)	0.10 (0-0.75)	24.6	0.027
IL8	6.26 (2.2-17.18)	1.53 (0-3.1)	4.1	0.028
XCL1	1.41 (0.87-8.6)	0.20 (0-0.62)	7.2	0.028
AK3L1	0.25 (0.04-0.57)	0.62 (0.43-1.3)	2.46*	0.029
CCL11	9.15 (4.38-25.08)	1.82 (0.7-2.8)	5.0	0.029
TNFSF13B	9.67 (1.92-21.63)	2.14 (0.53-5.16)	4.5	0.029
VCAM1	4.77 (2.25-7.74)	1.27 (1.06-2.99)	3.8	0.029
CCL20	101.88 (21.46-429.74)	13.82 (6.72-21.71)	7.4	0.03
ITGAM	6.02 (2.16-16.52)	0.84 (0-2.62)	7.2	0.03
LTA	0.35 (0.06-1.58)	0.00 (0-0.13)	3520.0	0.033
TGFB3	1.00 (0.3-2.52)	0.18 (0-0.5)	5.6	0.033
NLRP1	1.62 (0.35-2.91)	0.18 (0-1.3)	8.8	0.034
CD14	3.08 (1.12-6.83)	0.90 (0.37-2.1)	3.4	0.036
CFLAR	2.23 (1.13-3.7)	1.09 (0.79-1.41)	2.1	0.036
CXCR4	4.07 (0.95-8.13)	0.99 (0.36-1.92)	4.1	0.036
GZMK	2.74 (0.78-9.52)	0.51 (0.1-1.48)	5.3	0.036
IKBKB	1.51 (0.64-2.51)	0.66 (0.5-0.74)	2.3	0.036
IL18	1.76 (0.64-3.19)	0.52 (0.35-1.47)	3.4	0.036
IL2RG	2.37 (0.67-4.42)	0.69 (0.09-1.4)	3.4	0.036
TLR2	4.09 (1.74-10.31)	1.17 (0.88-2.78)	3.5	0.036
PTGDR	2.51 (0.81-6.3)	0.31 (0.15-0.78)	8.0	0.037
CXCL2	4.69 (2.15-31.92)	1.14 (0.6-2.54)	4.1	0.039
TGFBR1	1.59 (0.63-3.32)	0.30 (0-0.82)	5.4	0.039
CD80	205.00 (72.83-326.9)	45.57 (12.7-72.23)	4.5	0.042
BCL2L11	1.77 (0.89-4.26)	0.70 (0-1.21)	2.5	0.043
BID	3.97 (1-13.7)	0.89 (0.55-1.31)	4.5	0.043
CD40LG	0.99 (0.3-2.03)	0.23 (0.11-0.77)	4.4	0.043
CKLF	5.26 (2.75-16.27)	1.67 (1.29-5.86)	3.2	0.043
LTA4H	1.78 (0.97-3.1)	0.90 (0.72-1.59)	2.0	0.043
TBK1	2.40 (0.92-3.66)	0.95 (0.69-1.56)	2.5	0.043
LAMP3	2.79 (0.42-5.79)	0.06 (0-0.82)	46.8	0.045
CD3E	2.18 (0.51-3.35)	0.46 (0.11-1.05)	4.8	0.047
TP53	1.84 (1.48-6.29)	0.93 (0.77-1.85)	2.0	0.047

n-fold nonprogressors vs. progressors, *1/n-fold.

Fig. 2. Prediction of graft function deterioration. Receiver operator characteristic (ROC) curve for graft function deterioration using multiple regression analysis of 5 the most significant gene variables indicates receiver operator characteristic area under the curve (AUC); sensitivity defines true-positive results and 100- specificity false- positive results. The area under the ROC curves was 0.878. Selected RQ cut-off value was 1.46 (85% sensitivity, 83% specificity).

subclinical inflammation by evaluation of molecular phenotype. Gene expression pattern of subclinical and clinical rejection was found to be different. Transcriptional profile of clinical inflammation showed stronger proinflammatory setting compared to subclinical inflammation. Moreover, trend of gradually increasing proinflammatory transcriptome rising from control group, escalating through subclinical and culminating in clinical inflammation group was observed. In other words, subclinical rejection samples showed an intermediate level of inflammatory transcripts in between normal and CI samples.

The quantitative differences seen in transcriptome of SCI and CI are consistent with the theory that subclinical rejection represents an early stage of TCMR and might progress to its clinical phenotype accompanied with graft function deterioration if left untreated. On the contrary, some believe that subclinical infiltration represent a self- limited *forme fruste* TCMR or injury- repair response to implantation injury rather than true TCMR [21]. However, their observations were based on earlier protocol biopsies performed at 6 weeks posttransplantation when the presence of disturbances in allograft caused by ischemia/reperfusion injury is more likely than in 3 months

Fig. 3. Immunohistochemical evaluation. Higher intensity of tubular PTGER2 protein staining in nonprogrisors (B) compared to progrisors (A).

protocol biopsies performed in our study. To exclude that stronger proinflammatory setting of CI in our study is not related to severe grades of rejection, we looked for correlations among the differential gene transcripts and histological inflammation scores and found only weak relationship with tubulitis and interstitial inflammation but no relationship with glomerulitis, intimal arteritis and grade of intragraft inflammation.

Some authors argue that subclinical infiltrates might represent injury-repair response without the functional consequences [21]. The higher incidence of delayed graft function observed in our subclinical inflammation group might be suspected of “injury-repair response” nature of silent T- cell mediated inflammation rather than of the true TCMR. However, no association between differential gene expressions and DGF was found which favoured our decision not to exclude DGF grafts from the analysis.

We are aware of fact that borderline changes are not classified by Banff classification as conventional TCMR and their inclusion into studied groups might represent a potential disturbance when interpreting the results of our study. However, subclinical TCMR and borderline changes have showed an identical molecular phenotype [21]. Moreover, some observational studies have shown that persistent inflammation even below the Banff threshold diagnosis for acute rejection, results in worse graft function [29]. Furthermore, the treatment of borderline change is recommended by KDIGO [23]. After all, inter- and intra-observer agreement on the Banff classification grade, especially on borderline lesions, varies significantly among different centres [4, 5].

One might argue that the long-term advantage of treatment of subclinical rejection is controversial. However, evidence favours treating subclinical rejection [23]. It is known, that early subclinical inflammation represents a risk factor for the development of interstitial fibrosis [5, 30] and persistent inflammation is associated with fibrosis progression and chronic antibody-mediated rejection [31, 32]. It was also shown that subclinical antibody-mediated rejection is associated with an increased risk of graft loss, irrespective of the kidney allograft function at the time of biopsy [33]. All above mentioned supports the importance of early recognition and surveillance of subclinical inflammation.

We also aimed to validate the applicability of molecular analysis of renal allograft in predicting the graft function deterioration. Some studies suggest that molecular phenotype of protocol biopsies has little relationship to future events and outcomes [21] while the others report findings of potential molecular predictors [22, 34]. In our study, multiple logistic regression analysis revealed that low prostaglandin E2 receptor (PTGER2) gene expression levels were predictive of graft function deterioration or vice versa, higher PTGER2 gene expression level might be associated with protection against progression of renal dysfunction. Prostaglandin E2 (PGE2) is mediating a variety of both innate and adaptive immune responses through 4 distinct receptors: EP1R to EP4R and is widely viewed as a general immunosuppressant [35-37]. It has been reported that PGE2 significantly inhibit allograft rejection in animal transplantation models [38] and exerts its immunosuppressive properties through EP2 and EP4 receptors [39]. EP2R and EP4R agonist might, through the modulation of immune responses, including IFN- γ production, inhibition of up-regulation of adhesion and costimulatory molecules represent potential targets for immunosuppressive strategies [40-43]. EP2 receptors directly inhibit T cell proliferation and regulate antigen presenting cells functions [44], inhibit IL-5 and IFN- γ production, suggesting suppression both Th1- and Th2- antigen-specific human T-cell responses [45].

Moreover, EP2 and EP4 agonists significantly prolonged cardiac allograft survival in a murine cardiac transplantation model [39]. EP4 agonist was more effective than the EP2 agonist to inhibit acute allograft rejection, suggesting more important role of EP4. Some exciting data have emerged concerning the EP4 selective agonist as a novel agent to protect both posttransplantation inflammatory and alloimmune responses [42, 46-48]. However, in our study we were not able to assess the importance of EP4 receptor in progression of renal dysfunction in kidney allografts with findings of subclinical or clinical inflammation as the PTGER4 gene was not included in the evaluated gene set. Although PGE2 has recently emerged as an immunoactivator through facilitation of Th1 differentiation and Th17 expansion [49, 50], the vast majority of studies still report the traditional belief that EP2-EP4 agonists [40, 43], not antagonists, can be a suitable therapeutic drug target for inflammation diseases. We believe that PGE2 receptors might merit further study as novel therapeutic for clinical transplantation.

Although we were able to detect higher expression of Treg-specific cell marker (HPGD) in SCI samples, no enhancement of FoxP3 expression was seen. The low expression of FoxP3 in SCI, despite the pure subclinical setting is surprising and contradictory to previous publications, where it was reported as being high, especially in borderline changes [51]. The difference can be explained in part by the inclusion of severe grades of subclinical rejection.

However, our study was not designed to show differences in renal allograft transcriptome between different rejection grades so data should not be over interpreted. Decreased expression of KNG1 in CI compared to SCI and control is fully consistent with literature knowledge of decreased kininogen1- proteomic activity in acute cellular rejection [52].

Although protocol biopsies are routinely performed almost in all transplant centres, no uniform opinion on benefit of SCI treatment exists. Furthermore no convincing predictors of graft function deterioration are known. While histological evaluation of renal biopsy remains the current “gold standard” for assessment of acute rejection, molecular analysis might help to understand the background running processes and contribute to elucidate the silent course of subclinical rejection. Some authors believe that histological findings precede functional deterioration. We and others even believe that molecular response precedes histological changes [53, 54]. Hence, we chose to compare the molecular phenotype of subclinical inflammation in 3 month protocol biopsies with clinical inflammation in early case biopsies. Although the study was limited by the sample size and its descriptive nature, our preliminary findings provide interesting information on transcriptional difference between subclinical and clinical rejections. The degree of alloimmune inflammation gene expression in subclinical inflammation was milder than in clinical inflammation group suggesting the explanation for different clinical course of subclinical rejection

Conclusion

The presented results might provide additional proof that subclinical acute inflammation has transcriptional profile of acute injury of lower extent than clinical inflammation. This study also supports the concept that rejection is a linear process with the clinically detectable rise in creatinine being the latest event what indirectly emphasizes the importance of early treatment of subclinical rejection to prevent progression to more intense alloimmune activation and development of interstitial fibrosis. If the PTGER2 gene expression is as a potential indicator of graft function deterioration must be validated in further studies.

Abbreviations

AUC, area under curve; BNIP3L, BH3-only protein family; CASP, caspase; CCL, chemokine ligand; CCR, chemokine receptor; CCRL, chemokine receptor-like; CD, cluster of differentiation; CSF, colony stimulating factor; CXCR, chemokine receptor; CI, clinical inflammation; DGF, delayed graft function; EP, prostaglandin receptor; HPGD, hydroxyprostaglandin dehydrogenase; IL, interleukin; IL1RAPL2, interleukin 1 receptor accessory protein-like 2; IFN- γ , interferon gamma; KDIGO, kidney disease improving global outcomes; KNG1, kinogen-1; LAMP3, lysosome-associated membrane glycoprotein 3; LILRB, leukocyte Ig-like receptor; LTA, lymphotoxin A; MAPK 14, mitogen-activated protein kinases; MMF, mycophenolate mofetil; NFKBIE, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon; PGE2, prostaglandin E2; PTAFR, platelet-activating factor receptor; PTGER2, prostaglandin E2 receptor; ROC, receiver operating characteristic; RQ, relative quantification; RT-PCR, reverse transcriptase polymerase chain reaction; SCI, subclinical inflammation; TAC, tacrolimus; TCMR, T-cell mediated rejection; TGFA, transforming growth factor alpha; TGF- β , transforming growth factor β ; TLDA, Taqman® low density array

Conflict of Interest

The authors have declared no conflicts of interest.

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