A Computational Gene Expression Score for Predicting Immune Injury in Renal Allografts

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Abbreviations: AR, acute rejection; no-AR, no acute rejection; IFTA, interstitial fibrosis tubular atrophy; pIFTA, progressive interstitial fibrosis tubular atrophy; P, progressors; NP, Non-progressors; CRM, common immune response module; tCRM, tissue common

Abstract

Background

Whole genome microarray meta-analyses of 1030 kidney, heart, lung and liver allograft biopsies identified a common immune response module (CRM) of 11 genes that define acute rejection (AR) across different engrafted tissues. We evaluated if the CRM genes can provide a molecular microscope to quantify graft injury in acute rejection (AR) and predict risk of progressive interstitial fibrosis and tubular atrophy (IFTA) in histologically normal kidney biopsies.

Methods

Computational modeling was done on tissue qPCR based gene expression measurements for the 11 CRM genes in 146 independent renal allografts from 122 unique patients with AR (n = 54) and no-AR (n = 92). 24 demographically matched patients with no-AR had 6 and 24 month paired protocol biopsies; all had histologically normal 6 month biopsies, and 12 had evidence of progressive IFTA (pIFTA) on their 24 month biopsies. Results were correlated with demographic, clinical and pathology variables.

Results

The 11 gene qPCR based tissue CRM score (tCRM) was significantly increased in AR (5.68 ± 0.91) when compared to STA (1.29 ± 0.28; p < 0.001) and pIFTA (7.94 ± 2.278 versus 2.28 ± 0.66; p = 0.04), with greatest significance for CXCL9 and CXCL10 in AR (p <0.001) and CD6 (p<0.01), CXCL9 (p<0.05), and LCK (p<0.01) in pIFTA. tCRM was a significant independent correlate of biopsy confirmed AR (p < 0.001; AUC of 0.900; 95% CI = 0.705–903). Gene expression modeling of 6 month biopsies across 7/11 genes (CD6, INPP5D, ISG20, NKG7, PSMB9, RUNX3, and TAP1) significantly (p = 0.037) predicted the development of pIFTA at 24 months.

immune response module; qPCR, quantitative polymerase chain reaction; cDNA, complementary DNA; Ct, comparative threshold cycle; ROC, Receiver Operating Characteristic; ANOVA, analysis of variance; SEM, standard error of mean.

Conclusions

Genome-wide tissue gene expression data mining has supported the development of a tCRM-qPCR based assay for evaluating graft immune inflammation. The tCRM score quantifies injury in AR and stratifies patients at increased risk of future pIFTA prior to any perturbation of graft function or histology.

Introduction

Kidney transplantation is the preferred modality for treatment of end-stage renal disease by any cause [1] and leads to better outcomes than dialysis [2]. However, long-term kidney allograft outcomes have not improved as expected despite a better understanding of the immune biology of allograft rejection and the advent of novel and more potent immunosuppressive agents [3]. Chronic allograft nephropathy continues to be the main reason for poor outcome and loss of graft and may be attributed to poor immune-risk assessment of transplant patients in current clinical practice. The main metrics used for monitoring a renal allograft are the relatively insensitive surrogate markers of allograft dysfunction such as serum creatinine [4, 5] as well as the use of allograft biopsies to directly diagnose histological lesions that are consistent with either acute rejection or interstitial fibrosis and tubular atrophy (IFTA). However, the serum creatinine increases due to many other reasons not related to allograft rejection such as immunosuppressive drug-related nephrotoxicity, urinary infections, or dehydration. The drift in serum creatinine is not predictive of tissue injury as the increase is seen late in injury, once allograft damage is already established; hence it has no utility for modifying treatment for prevention of rejection and/or IFTA. Furthermore, while the use of surveillance biopsies has been postulated as the gold standard tool for diagnosing allograft lesions, this approach is costly and invasive, even requiring sedation, particularly among pediatric transplant patients [6]. In addition, we and other have shown that immune injury predates chronic damage [7–10]. In a previously published paper we reported a common rejection module (CRM) consisting of 11 genes that were significantly overexpressed in acute rejection (AR) across all transplanted organs. The meta-analysis of eight independent transplant datasets from four organs yielded the CRM genes that could diagnose AR with high specificity and sensitivity in five additional independent cohorts [11]. In this study have analyzed the 11 CRM genes for their value as biomarker panel to diagnose AR and predict risk of accelerated or progressive IFTA injury (pIFTA). We sought out to validate the molecular changes within the allograft before and during acute rejection injury and evaluated if the combined expression of a finite set of the 11 CRM genes.

Materials and Methods

Study samples

All patients included in the study gave written informed consent to participate in the research, in full adherence to the Declaration of Helsinki. The study was approved by the institutional review board at Stanford University and University of California San Francisco. 146 renal allograft biopsies from 122 unique renal transplant patients were collected between 1 month– 10 years post-transplant as protocol biopsies or as indicated by acute graft dysfunction from pediatric and adult renal transplant patients with stable renal function (no-AR), AR, and pIFTA (for demographics see Table 1). Patients with acute rejection had biopsies collected prior to treatment intensification. Diagnosis of AR and IFTA was made by biopsy histology Banff

Table 1. Demographic Table.

Main demographics	AR(n = 54)	no-AR(n = 56)	pIFTA (n = 12)
Recipient gender (F, %)	20 (37)	25 (45)	5 (42)
Recipient age (years)	9.5±4.7	10.7±5.4	11.4±5.7
Donor gender (F, %)	26 (47)	20 (35)	6 (50)
Donor age (years)	27.9±13.6	30.0 ±11.5	33.5±6.8
Recipient Race (% Caucasion/Asian/Hispanic/African-American)	60/20/15/5	58/20/20/2	55/18/19/2
Type of Transplant (%)-Living	9 (17.3)	6 (10.5)	1 (8.3)
Type of Transplant (%)-Living-related	2 (3.8)	10 (17.5)	4(33.3)
Type of Transplant (%)-cadaver	7 (13.4)	19 (33.3)	2 (16.7)
*Cause of ESRD (%) (1/2/3/4/5/6)	10/5/8/9/6/16	14/5/5/8/8/16	2/0/3/2/2/3
Serum Creatinine (mg/dL) (mean+/- SD)	1.45±0.88	1.50±0.79	0.97± 0.0
post-transplantation (month)	33.99±31.58	14.00±9.15	11.32±9.91

*ESRD (1/2/3/4/5/6): 1, glomerulonephritis, 2, polycystic kidney disease, 3, renal dysplasia, 4, reflux nephropathy, 5, obstructive uropathy, 6 = other or unknown. None of these selected patients had delayed graft function. 20% of AR episodes were associated with documentation of non-adherence with medications (self-reported), appointments and/or laboratory measurements. Though the time post-transplant was significantly greater for AR (p<0.05); there was no difference in post-transplant time between no-AR and pIFTA.

classification [12]. All AR were T-cell mediated or mixed with T cell and antibody mediated injury (grade IA or higher) and pIFTA samples showed Banff scores grade II or higher (II and III), without showing any other specific accompanying lesions or AR. 1/3 of a needle biopsy core was collected in RNAlater solution and stored at -20°C until RNA extraction for the qPCR studies.

Patient demographics

This study used a total of 146 independent renal allografts collected from 122 unique patients with biopsy proven AR (n = 54) and no-AR (n = 92). 24 demographically matched patients with no-AR had 6 and 24 month paired protocol biopsies. Among these 24 patients all of them had histologically normal biopsies in 6 month post-transplantation time. However, only 12 of the 24 patients had evidence of progressive IFTA (pIFTA) on their 24 month biopsies. The remaining 12 patients were normal in their 24 month post-transplantation. The cross sectional samples were randomly split into two groups of 27 AR and 22 no-AR for the purpose to determine and validate a tCRM threshold for detection of AR. Of this latter group demographic variables were matched. For the 24 patients with paired samples everyone had normal kidney function and graft histology on the 6 month biopsies. Twelve patients in this group had pIFTA injury on their 24 month protocol biopsies (also labeled as progressors or P). The remaining 12 patients had histologically normal 24 month protocol biopsies (also called non-progressors or NP) (Table 1). All the patients received a calcineurin-inhibitor immunosuppressive regimen based on tacrolimus and induction therapy either with a T-cell depleting agent (Thymoglobulin) or with an anti-IL2 receptor monoclonal antibody (basiliximab). There was no statistical significance in between the demographical and the clinical parameters.

Total RNA extraction, cDNA synthesis and qPCR

Total RNA was extracted from each biopsy using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA integrity was ensured using the RNA 6000 NanoLab Chip Kit (Agilent Technologies). cDNA synthesis was performed using 25 ng of extracted quality total RNA from the biopsy

samples using SuperScript VILO Master Mix (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. Briefly, specific target amplification was performed using 3.125 ng relative amount of cDNA using a pooled individual TaqMan real-time assays for the 11 genes investigated in multiplex with TaqMan PreAmp Master mix Life Technologies) to 10 μl final volume, for 18 cycles in a thermal cycler (Eppendorf Vapo-Protect, Hamburg, Germany), then diluted 1:20 with sterile water (Gibco, Invitrogen, Carlsbad, CA). The qPCR was performed on the QuantStudio 6 Flex System (Life Technologies) using 5μl of the diluted sample from the specific target amplification, along with the TaqMan Gene Expression Master Mix under standard conditions (2 min at 50°C, 10 min at 95°C, 40 cycles of 15s at 95°C, 1min at 60°C) using TaqMan gene expression assays (Life Technologies) for each of the 11 genes investigated: BASP1, CD6, CXCL10, CXCL9, INPP5D, ISG20, LCK, NKG7, PSMB9, RUNX3, TAP1. The relative amount of mRNA expression in each sample was calculated using the comparative threshold cycle (C_T) method [13]. Ribosomal 18S RNA (18S) and Universal RNA (QIAGEN) were used for normalization of all genes since they showed the least variability in gene expression across all samples. Final gene expression results were converted to fold change. tCRM score was calculated by using the geometric mean of the fold changes of the respective genes.

Data analyses

All data are presented as mean ± standard error of the mean (SEM). Groups were compared using the χ^2 test for categorical variables, the two-way analysis of variance (ANOVA) or t-test for normally distributed data, and the nonparametric Kruskal–Wallis, Welch's correction or Mann–Whitney U test for non-normally distributed variables. Bivariate correlation analyses were done using Pearson or Spearman tests for non-parametric variables. Sensitivity/specificity Receiver Operating Characteristic (ROC) curve analyses were performed to evaluate the most precise cut-off of the tissue CRM (tCRM) gene scores assessed at the time of biopsy, predicting the advent of acute rejection (AR). All predictive values were determined by calculating the area under the curve with SPSS software. Binary linear logistic regression analysis was performed to determine the independent correlation of several independent variables with the presence of AR. The statistical significance level was defined as $p<0.05$. Gene expression, tCRM scores and clinical variables were examined by multivariate analysis to determine predictive value. Cross sectional analysis was done to determine the significant increase in gene expression at event. Longitudinal studies were done to determine changes in gene expression over 2 years in patients with progressive IFTA (pIFTA).

Results and Discussion

The 11-gene intra-allograft common response module (tCRM) score accurately segregates acute rejection (AR) from stable (no-AR) kidney transplant patients in kidney tissue biopsies

The individual expressions of all 11 genes of the tCRM score showed a significant increase in AR, with the most significant increase seen in CXCL9 and CXCL10 (p = 0.0001 in both comparisons) between AR and no-AR (Fig 1). The tCRM score was also significantly increased in AR (mean ± SEM = 6.897 ± 1.082, n = 27) when compared to no-AR
(mean ± SEM = 0.8144 ± 0.1374, n = 22, p = 0.000057) (Fig 1), and increased for pIFTA cohort for both the 6 and 24 month biopsies over the biopsies from patients who did not develop any histological changes (3.33 ± 1.00 versus 1.22 ± 0.2 at 6 months, p = 0.05; 7.94 ± 2.281 versus 2.28 ± 0.66 at 24 months; p = 0.03 at 24 months respectively) (Fig 1). Using the first set of 49 biopsies (27AR, 22no-AR), the tCRM score was found to be a significant correlate of Banff
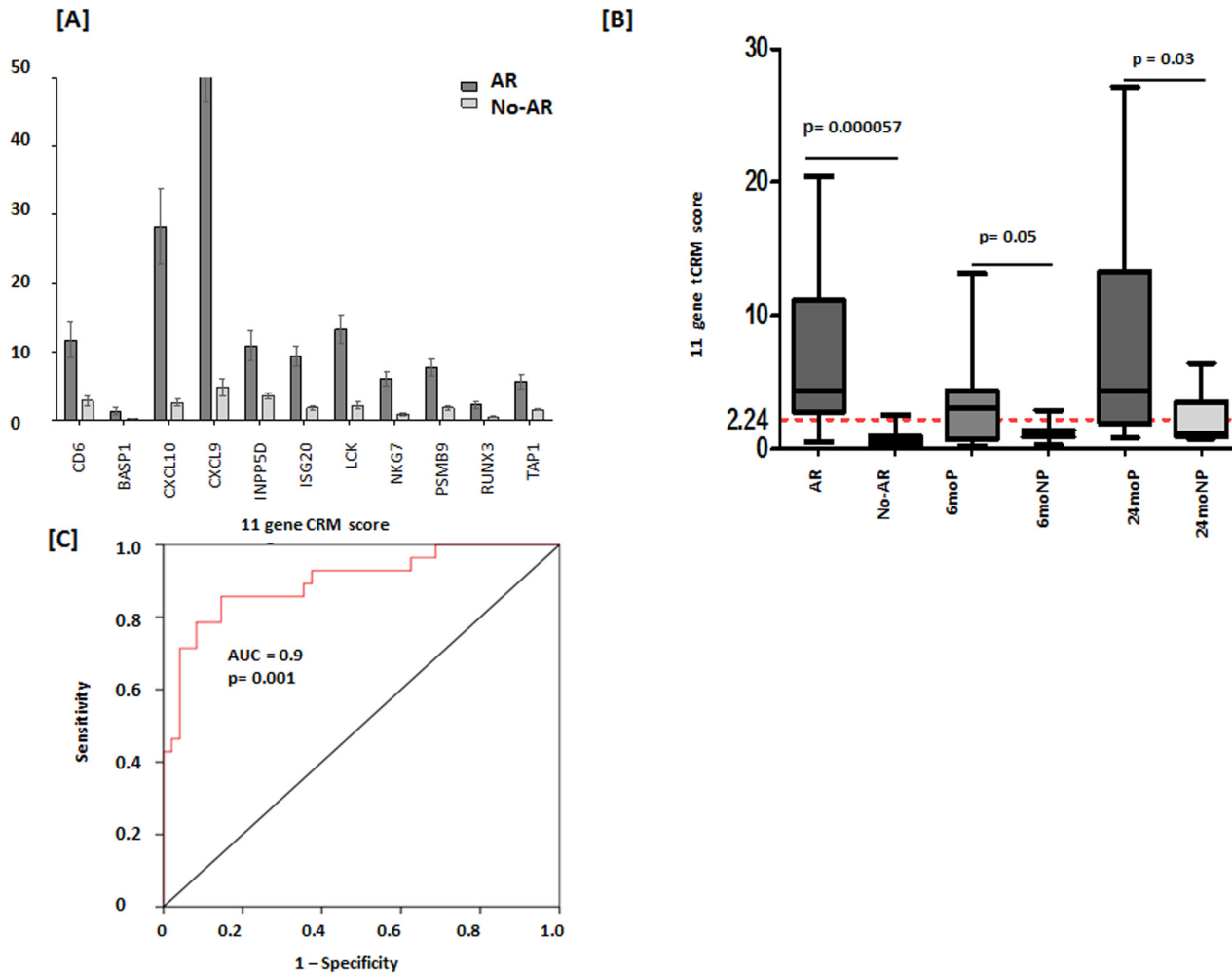
Fig 1. Gene expression of tCRM genes in AR. [A] Intra-allograft gene expression for tCRM genes increased in AR when compared to stable with the most significant differences seen between CXCL10 and CXCL9 (p = 0.0001). [B] The tCRM score is significantly increased in AR when compared to Stable (p = 0.0000057). Although there is increased in pIFTA progressors at 6 months, this difference did not meet significance (p = 0.05) until 24 months (p = 0.03). A threshold tCRM score of 2.24 as determined by the discovery set. [C] The tCRM score significantly predicts rejection with an AUC of 0.900 (p = 0.001, 95% CI = 0.823–0.976) with 82.7% sensitivity and 82.5% specificity.

classified AR (AUC of 0.900 (p <0.001, 95% CI = 0.823–0.976) (Fig 1). With the aim of using the tCRM score as a binary variable to segregate patients at AR risk based on the tCRM score alone, the data was analyzed to choose a tCRM score of 2.24 which resulted in the greatest sensitivity and specificity for AR prediction (sensitivity = 82.7%, specificity = 82.5%) (Fig 1). When applied to the second independent biopsy set of 49 biopsies (27AR, 22no-AR) set, the tCRM threshold of 2.24 had a positive predictive value (PPV) for AR of 82.4%.

The tCRM score correlates with the extent of AR lesions

We evaluated whether the tCRM score was associated with the extent of the AR lesions observed in the matched biopsies. As observed in Fig 2, the tCRM score strongly correlated with the extent of the acute allograft lesions both at the tubular (Banff t score and the interstitum (Banff i score) kidney compartments (R = 0.72, p = 0.001 and R = 0.74, p = 0.001).
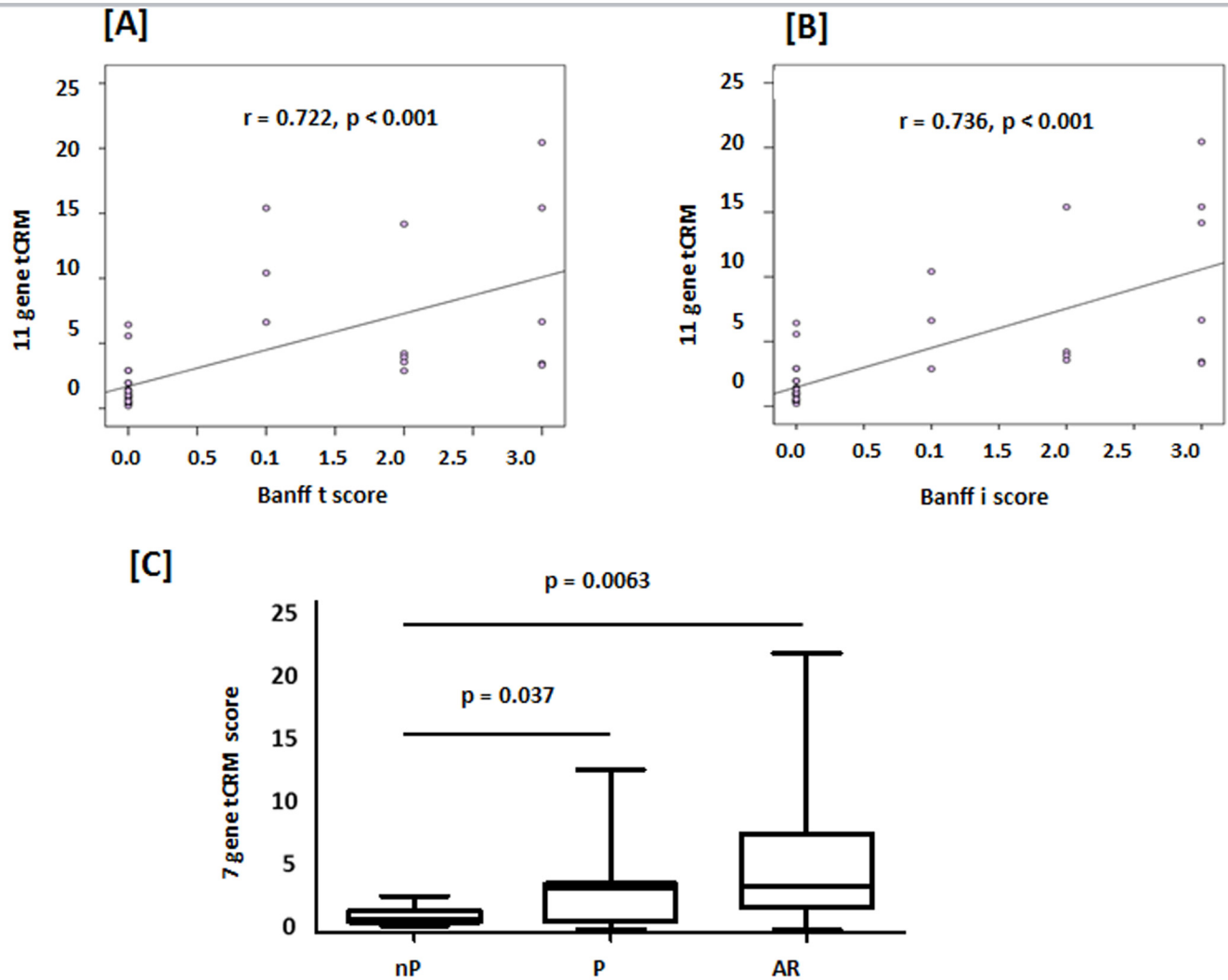
Fig 2. tCRM score correlates with AR lesions and chronic allograft nephropathy. [A] The tCRM score positively correlates significantly (p = 0.001) with the degree of infiltrates found on biopsy for the t-score = 0.722 and [B] i score = 0.736. [C] A tCRM score across a subset of 7 of the 11 genes differentiated most samples with pIFTA or progressors (3.29 ± 0.93) from no-AR patients (1.2 ± 0.18; p = 0.037). Stable/non-progressors (NP) and AR were highly distinguishable (1.198±0.1801 versus 5.582±0.8651; p = 0.0063). pIFTA/Progressors and AR were not different with regards to their tCRM scores (p = 0.16).

doi:10.1371/journal.pone.0138133.g002

The tCRM score is predictive of chronic allograft nephropathy

Biopsies from patients with chronic allograft changes as defined by the presence of IFTA were examined at 24 months, and in addition their biopsy pairs were also examined from their 6 month protocol biopsies. Significantly increased fold changes of all genes except BASP1 and CXLC10 were seen at both 6 and 24 months between pIFTA (P) and no-AR (NP) patients. While the tCRM score across all 11 genes was significant between pIFTA (P) and no-AR (NP) (p = 0.05), we found by using an adjusted R score analysis, that among the 11 genes, the greatest influence on pIFTA was from a subset of 7 genes (CD6, INPP5D, ISG20, NKG7, PSMB9, RUNX3, and TAP1), with maximal influence from CD6. A modified tCRM score across these 7 genes showed significantly greater scores in the 6 month biopsies of patients destined to develop pIFTA patients over time (p = 0.037) (Fig 2).

Discussion

There is an urgent need in transplant medicine for developing reliable and non-invasive monitoring tools that may help transplant clinicians predict the risk of alloimmune-mediated allograft injury, preferentially before allograft damage has already been established. While a number of transcriptional biomarkers have been associated to AR [14–19], many studies either have limited sample sizes [20–23] or have focused on exploratory single biomarkers that may not necessarily reflect the crux of the molecular complexity in allograft rejection [24, 25]. Taking advantage of recently reported microarray meta-analyses of eight independent transplant datasets by combining effect size and p-values from our group [11], showing a common immune response module (CRM) of gene expression in allograft biopsies during AR, irrespective of the type of tissue organ, the main goal of this study was to validate these findings using the more practical method of PCR as well as determine a threshold score. We found that a qPCR-based intra-allograft tCRM score threshold of >2.24, can accurately distinguish AR from no-AR with high sensitivity and specificity. This data allows us to confirm the association of gene expression across this restricted set of genes with the histological immune injury of acute rejection, and also supports our earlier findings [7] that the molecular profile of immune injury is a threshold effect, with greater burden of injury in acute rejection, and similar, but lower injury burden in the subclinical injury of progressive IFTA. The panel of genes has differential impact on the injury phenotypes such as CXCL9 and CXCL10 are important layers in the injury of AR, and CD6 plays a major role in pIFTA, suggesting different roles for cell infiltration versus activation in acute and chronic graft injury. Cytokines are involved in all inflammatory responses. Being a class of small cytokines, the C-X-C motif chemokine ligand CXCL9 and CXCL10 play key roles in the initiation and development of acute transplant rejection [26]. Our observation of CXCL9 and CXCL10 being important factor in AR corroborates previously reported observations regarding their involvement in immune mediated graft injury [14–16, 19]. Our ongoing studies show the tCRM score is also elevated in BK viral nephritis in the graft (Sigdel *et al*, in submission), which supports data from previous reports of significant overlap in the molecular disturbances from the overlapping intragraft infiltrates in AR and BK viral nephritis [14, 27–30], though in the latter instance the diagnosis of BK viral infection is very evident also by BK viral urine and blood PCR positivity and tissue SV40 positive stains. Nevertheless, in histologically normal biopsies, and in the absence of AR and confounding intragraft infection, an elevated tCRM score is a strong harbinger of underlying molecular inflammation and can provide a very important warning to a clinician to closely monitor this patient for risk of pIFTA. Recent years have seen an increased effort to develop and validate new assays for early diagnosis of acute rejection of organ transplantation and transplant monitoring [31]. We and others have reported on potential surrogate gene biomarkers for acute rejection analyzing peripheral blood [32, 33], gene biomarkers using urine [34–36] and protein biomarkers analyzing serum and plasma [37], and urine [10, 38–41]; many of these are in varying stages of further validation. We suggest that the inclusion of the tCRM score into the analysis of the immune profile of protocol biopsies can be very valuable for risk analysis in the context of clinical trials, as the tCRM score may provide a companion diagnostic for differentiating patients into high and low immune risk, for stratification into different investigative treatment arms, with an increased margin for patient and graft safety. Because of the restricted sample size available for this study, we suggest that the value of the tCRM score to discriminate patients at increased risk of AR or IFTA, prior to any perturbation of graft function or histology may require further validation in a larger cohort of patients.

Acknowledgments

Author Contributions

Conceived and designed the experiments: MMS TKS OB. Performed the experiments: TQT SCH ID. Analyzed the data: TKS OB MMS SR. Contributed reagents/materials/analysis tools: FV. Wrote the paper: TKS OB MMS.

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