Urinary granzyme A mRNA is a biomarker to diagnose subclinical and acute cellular rejection in kidney transplant recipients

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The distinction between T-cell-mediated rejection (TCMR) and other causes of kidney transplant dysfunction such as tubular necrosis requires biopsy. Subclinical rejection (SCR), an established risk factor for chronic allograft dysfunction, can only be diagnosed by protocol biopsy. A specific non-invasive biomarker to monitor immunological graft status would facilitate diagnosis and treatment of common transplantation-related complications. To identify possible markers, we measured urinary mRNA levels of several cytolytic proteins by quantitative PCR. Our cohort of 70 renal transplant recipients had biopsy proven type I and type II TCMR, acute tubular necrosis, SCR, calcineurin inhibitor-toxicity, cytomegalovirus infection, and stable graft function with normal histology. Granzyme A (GzmA) mRNA was significantly higher in subclinical and acute cellular rejection compared to patients with stable grafts or those with tubular necrosis with 80% sensitivity and up to 100% specificity. Granzyme B and perforin mRNA levels could significantly discriminate acute rejection from stable or tubular necrosis, but were not significantly elevated during SCR. Importantly, only GzmA mRNA remained below detection limits from grafts that were stable and most with tubular necrosis. Hence, the presented data indicate that urinary GzmA mRNA levels may entail a diagnostic non-invasive biomarker to distinguish patients with subclinical and acute cellular rejection from those with tubular necrosis or stable grafts.

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Subclinical[1] and acute T-cell-mediated rejection (TCMR)[2] are well described risk factors for late kidney graft failure. Improving late graft outcome is currently the major clinical challenge in transplantation. Timely detection and treatment of rejection are important to preserve long-term graft function. Standard diagnostics rely on serial serum creatinine measurements followed by histopathological analysis of a biopsy in case of increased creatinine. This strategy implies that rejection is diagnosed when immunological injury has led to impaired graft function. Subclinical rejection (SCR) is a silent form of inflammation that does not lead to immediate deterioration of function, and SCR diagnosis therefore requires planned protocol biopsies. Nevertheless, SCR is an important risk factor for chronic allograft dysfunction, the leading cause of late graft loss underscoring the importance of timely detection.[3–6] Several studies have shown that steroid treatment of SCR reduces chronic damage and favors better outcome.[7,8] However, these results are controversial.[9] Overall, studies that address long-term graft outcome after SCR treatment are lacking. Accurate diagnosis of both SCR and TCMR still needs biopsy. The invasiveness of this procedure and risk for complications preclude routine usage of repetitive biopsies. Additionally, sampling errors and inter-observer variability complicate accurate diagnosis.[10,11] To avoid biopsy, many attempts have been made to identify non-invasive rejection markers.[12] Yet, none have made their entry into clinical practice.

The intragraft presence of granzyme B (GzmB)-positive cytotoxic T lymphocytes and natural killer cells is associated with rejection.[13] Urinary mRNA levels of GzmB, perforin, GzmB inhibitor serpinB9 and FoxP3 have been put forward as parameters to diagnosis rejection.[14–16] Sensitivity of these biomarkers

Table 1 | Demographic and transplantation characteristics

Characteristics	Stable (n=10)	ATN (n=9)	CNI (n=4)	TCMR-I (n=20)	TCMR-II (n=7)	SCR (n=10)	CMV (n=10)	P-*value*
Recipient								
Age (median (year), quartiles)	44 (37–58)	57 (37–66)	52 (30–56)	53 (38–58)	47 (26–52)	51 (45–58)	46 (37–55)	0.86
Gender (% male)	70	56	50	80	57	80	70	0.80
Pretransplant CMV status (% positive)	50	78	75	60	86	50	40	0.42
Primary disease (number of patients, %)								0.50
Focal sclerosis	1 (10)	1 (11)	0	3 (15)	1 (14)	1 (10)	0	
Reflux nephropathy	1 (10)	0	0	2 (10)	1 (14)	0	0	
IgA nephropathy	2 (20)	0	0	6 (30)	2 (29)	3 (30)	4 (40)	
Polycystic kidney disease	4 (40)	1 (11)	0	1 (5)	0	1 (10)	2 (20)	
Glomerulonephritis	0	1 (11)	2 (50)	2 (10)	1 (14)	0	1 (10)	
Systemic autoimmune disease	0	0	0	1 (5)	0	0	0	
Diabetic nephropathy	0	0	0	1 (5)	0	0	0	
Hereditary disease	0	0	0	1 (5)	0	1 (10)	0	
Hypertension	0	4 (44)	2 (50)	2 (5)	2 (29)	4 (40)	1 (10)	
Uncertain etiology	2 (20)	2 (22)	0	1 (5)	0	0	2 (20)	
Transplantation type (%)								0.18
Deceased donor, heart beating	60	33	25	55	43	50	50	
Deceased donor, non-heart beating	10	67	25	20	43	10	20	
Living donor, related	20	0	0	5	14	20	10	
Living donor, unrelated	10	0	50	20	0	20	20	
Donor								
Age (median (year), quartiles)	53 (30–57)	40 (35–59)	57 (49–60)	48 (29–57)	49 (48–61)	46 (38–54)	51 (47–58)	0.67
Gender (% male)	40	44	50	40	67	40	30	0.79
CMV status (% positive)	60	11	75	50	57	70	80	0.08
HLA mismatches total (median, quartiles)	3.0 (0.8–3.0)	3.0 (2.0–4.0)	3.0 (0.8–4.5)	3.0 (2.0–4.0)	3.0 (2.0–3.0)	2.5 (0.8–4.0)	3.0 (2.8–4.3)	0.76
Class I mismatches	2.0 (0.8–2.3)	2.0 (1.5–3.0)	2.5 (0.5–3.0)	2.0 (1.8–3.0)	2.0 (2.0–3.0)	2.0 (0.8–2.3)	2.0 (1.8–3.0)	0.85
Class II mismatches	1.0 (0.0–1.0)	1.0 (0.0–1.0)	0.5 (0.0–1.8)	1.0 (0.0–1.0)	0.0 (0.0–1.0)	0.5 (0.0–1.3)	1.0 (0.8–1.3)	0.51
Cold ischemia (median (h), quartiles)	16 (5–22)	21 (18–26)	11 (2–20)	16 (4–23)	19 (13–22)	12 (3–17)	15 (4–20)	0.19
Delayed graft function (% present)	20	78	25	40	57	20	20	0.07
Characteristics at urine sampling								
GFR (median (ml per min per 1.73 m^2), quartiles)[a]	66 (49–71)	9 (6–29)	38 (28–40)	24 (10–28)	11 (7–17)	42 (31–53)	42 (25–62)	<0.01
Time of urine sampling (median (weak after TX), quartiles)	5 (1–16)	3 (2–4)	135 (51–204)	5 (1–8)	2 (1–5)	26 (14–30)	8 (4–9)	<0.01

Abbreviations: ATN, acute tubular necrosis; CMV, cytomegalovirus; CNI, calcineurin inhibitor; GFR, glomerular filtration rate; HLA, human leukocyte antigen; IgA, immunoglobulin A; MDRD, modification of diet in renal disease; SCR, subclinical rejection; TCMR-I, T-cell-mediated rejection type I; TCMR-II, T-cell-mediated rejection type II; TX, transplantation.
The different patient groups were compared by either Kruskal–Wallis tests for continuous variables or χ^2-tests for binary parameters.
[a]GFR in ml per min per 1.73 m^2 is calculated with MDRD study equation: $186 \times (\text{serum creatinine (mg/dl)})^{-1.154} \times (\text{age (year)})^{-0.203} \times (0.742, \text{if female}) \times (1.1210 \text{ if African Americans})$.[36]

to distinguish acute rejection from stable function varies between 79 and 100%, and specificity ranges from 64 to 90%, which impedes their use in immunological graft monitoring.

Granzyme A (GzmA) and GzmB are the most abundant cytolytic molecules of effector immune cells. However, these granzymes target distinct cell-death pathways.[17] Interestingly, recent studies have proposed an additional function for GzmA, triggering inflammation via induction of proinflammatory cytokines.[18–20]

In this study, we investigated the diagnostic value of GzmA mRNA in urinary cells as a diagnostic biomarker for subclinical and acute TCMR of kidney grafts.

RESULTS

Patient characteristics

The donor–recipient characteristics and transplantation parameters are detailed in Table 1. Biopsies were procured as protocol biopsies at 6 and 12 months after transplantation, upon clinical indication and according to the local standard of practice. All biopsies were scored using Banff '05 classification. On the basis of histopathological findings, 60 recipients were divided into six groups. Stable group consisted of patients with stable grafts with normal histology ($n=10$) at the time of sampling. Their 1-year post-transplant course was uncomplicated and 6 and 12 months biopsies showed normal histological findings. Other groups consisted of patients with acute tubular necrosis (ATN, $n=9$), calcineurin inhibitor toxicity ($n=4$), SCR ($n=10$), TCMR type-I (TCMR-I, $n=20$), and TCMR type-II (TCMR-II, $n=7$). Additionally, 10 patients with cytomegalovirus (CMV) infection were included.

Urinary GzmA mRNA expression is significantly elevated during rejection, both subclinical and acute TCMR-I

GzmA, GzmB, perforin, and SerpinB9 mRNA levels were measured in urinary cells (Figure 1, Supplementary Table S3 online). Gene expression was compared between stable grafts,

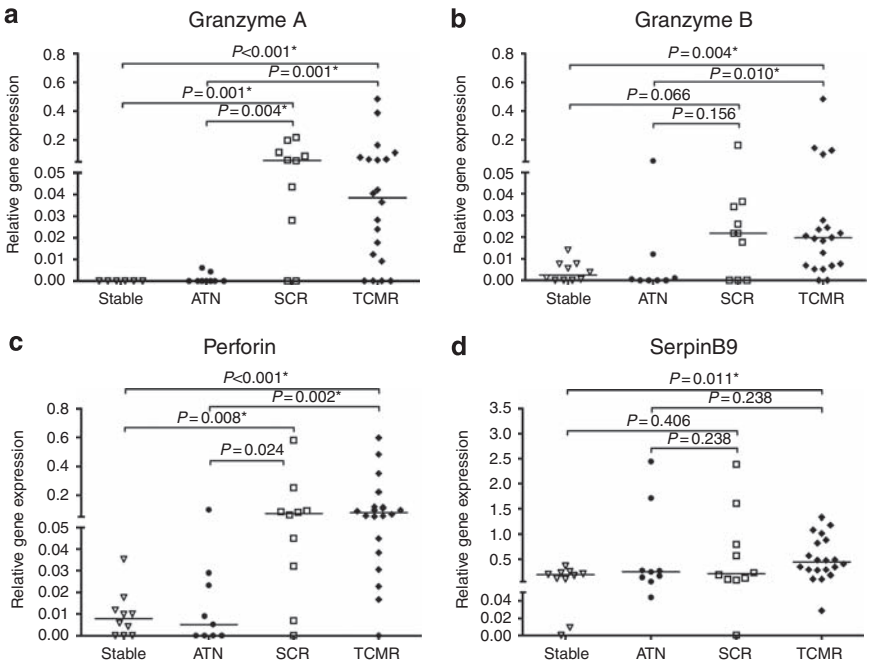


Figure 1 | Urinary granzyme A (GzmA) mRNA expression correlates with subclinical rejection and acute T-cell-mediated rejection (TCMR). Urine samples were collected from recipients with stable graft function (stable, $n=10$), acute tubular necrosis (ATN, $n=9$), subclinical rejection (SCR, $n=10$), and TCMR type I (TCMR-I, $n=20$). Transcription levels of GzmA, granzyme B (GzmB), perforin, and SerpinB9 were analyzed as described in Materials and Methods section. Horizontal lines represent medians, and asterisks refer to a significant difference (Mann–Whitney, $P<0.02$ after correction for multiple testing). (**a**) GzmA mRNA is significantly elevated in urine of both SCR and TCMR-I compared to stable graft function and ATN. (**b**) GzmB is significantly elevated during TCMR-I compared to stable and ATN. Despite a trend towards higher levels, GzmB is not significantly elevated during SCR compared to stable and ATN. (**c**) Perforin transcripts are measurable in all groups tested. Perforin levels are significantly increased during subclinical rejection (SCR) and TCMR-I as compared with stable. (**d**) SerpinB9 mRNA is commonly present in urine of transplant patients at high levels (note the high upper rank depicted on the *y* axis) and does not differ significantly between different groups. Transcription of this gene is significantly induced during TCMR-I compared with stable patients.

ATN, SCR, and TCMR-I. GzmA levels were significantly elevated in TCMR-I compared with stable grafts ($P<0.001$) and ATN ($P=0.001$). Interestingly, similar to TCMR-I, SCR correlated with measurable GzmA mRNA in contrast to undetectable levels found in stable grafts ($P=0.001$) and ATN ($P=0.004$) (Figure 1a).

In line with previous studies,[14–16] GzmB and perforin were significantly increased in TCMR-I as compared with stable grafts ($P<0.004$) and ATN ($P<0.010$) (Figure 1b and c). Relative high amounts of GzmB mRNA were found in patients with SCR. However, GzmB mRNA could not differentiate between SCR and either stable grafts ($P=0.066$) or ATN ($P=0.156$). Perforin levels were significantly higher in SCR than in stable grafts ($P=0.008$). Despite a trend, perforin expression was not significantly elevated during SCR as compared with ATN ($P=0.024$) after correction for multiple testing. SerpinB9 levels were generally high in all patients. Transcription of serpinB9 was increased during TCMR-I compared with stable grafts, just reaching the level of significance ($P=0.011$, Figure 1d). Expression did not differ significantly between any other groups.

In summary, GzmA was the only urinary biomarker that could distinguish both SCR and TCMR-I from stable grafts and ATN.

Transplantation-related complications can influence urinary gene expression

To address possible assay interference of common complications after kidney transplantations, the groups were extended with calcineurin inhibitor toxicity, TCMR-II, and CMV infection. Expression profiles of the calcineurin inhibitor toxicity samples were comparable with stable grafts and ATN, with GzmA below the detection limit and variable amounts of GzmB, perforin, and serpinB9 mRNA (Supplementary Table S3, Supplementary Figure S1 online). The low number of patients in this group hampers a valid statistical conclusion. GzmA and GzmB levels found during TCMR-II ($n=7$) were generally lower than those found in SCR and TCMR-I, and higher than stable grafts and ATN. None of these differences was significant. As GzmA mRNA was undetectable in most TCMR-II samples, this type of rejection may not be identified using GzmA.

The immune response against CMV might influence urinary gene expression. In our cohort, patients did not receive prophylactic antiviral treatment. Recipients with CMV infection showed a urinary gene-pattern comparable with TCMR-I and SCR. GzmA and perforin levels were significantly elevated during infection compared with stable grafts ($P<0.003$) and ATN ($P<0.010$).

In conclusion, calcineurin inhibitor toxicity, a common cause for diminished graft function, will unlikely interfere with GzmA as a marker to diagnose rejection. CMV infection can be a confounder, although infection is easily ruled out by routine CMV-PCR.

Urinary GzmA mRNA is a sensitive and specific biomarker for both subclinical and acute TCMR-I

To determine the diagnostic characteristics of GzmA, GzmB, perforin, and serpinB9, receiver operating curve analyses were performed (Figure 2a). Owing to undetectable GzmA in stable grafts and in the majority of ATN samples, the frequency of false-positive results was low. Urinary GzmA yielded a sensitivity of 80% and a specificity of 100% when used to distinguish both TCMR-I and SCR from stable grafts (Figure 2b, Supplementary Table S4 online). GzmA can discriminate SCR from ATN with a sensitivity of 80% and specificity of 100%. Comparing TCMR-I with ATN, GzmA reaches a sensitivity of 80% with a specificity of 78%.

The optimal cut-off values for GzmB and perforin result in reasonable sensitivities and specificities to distinguish both rejection types from ATN and stable grafts. However, their implementation as diagnostic markers is limited due to overlapping GzmB and perforin values measured in stable grafts, ATN, and during rejection (Supplementary Table S4 online).

Is GzmA an early biomarker for TCMR?

In 11 patients with TCMR-I, we could collect multiple urine samples with at least one sample before biopsy. In seven of these patients, GzmA was detectable before biopsy. GzmB was detectable before rejection in all patients, which is not remarkable considering the low GzmB expression found in 70% of patients with stable grafts. After rejection treatment and despite recovery of renal function to baseline levels, GzmA levels became undetectable in only 55% of the longitudinally followed up patients, but remained high or even increased in the others. CMV-PCR remained negative in all these cases. Unfortunately, the number of longitudinally followed up patients and the frequency of urine sampling is too low to determine whether GzmA precedes the rise in serum creatinine and whether persistent GzmA levels after treatment correlate with ongoing rejection.

Graft-infiltrating T cells express GzmA

Levels of urinary GzmA, GzmB, and perforin were significantly correlated within one urine sample ($P<0.001$, Spearman's rank correlation coefficients ranging from 0.59 to 0.72). In contrast, serpinB9, which is expressed in immune- and tubular epithelial cells,[21] did only mildly correlate with the other cytolytic genes. GzmA was the best marker to distinguish rejection from stable and ATN, suggesting selective expression of this protease. To identify the cell source of granzymes in kidney grafts, we performed triple immunohistochemical stainings: GzmA, GzmB, and CD3, on biopsies corresponding to tested urine samples ($n=3$ per group). Areas of $CD3^+$ and $CD3^-$ cells characterized both TCMR-I and SCR biopsies. Some of these infiltrating cells contained GzmA- and GzmB-positive granules (Figure 3). Although rare, we could detect low numbers of CD3-negative granzyme double-positive cells in TCMR-I and SCR biopsies. These findings confirm that mainly T cells but also natural killer cells are the source of urinary GzmA and GzmB mRNA.

DISCUSSION

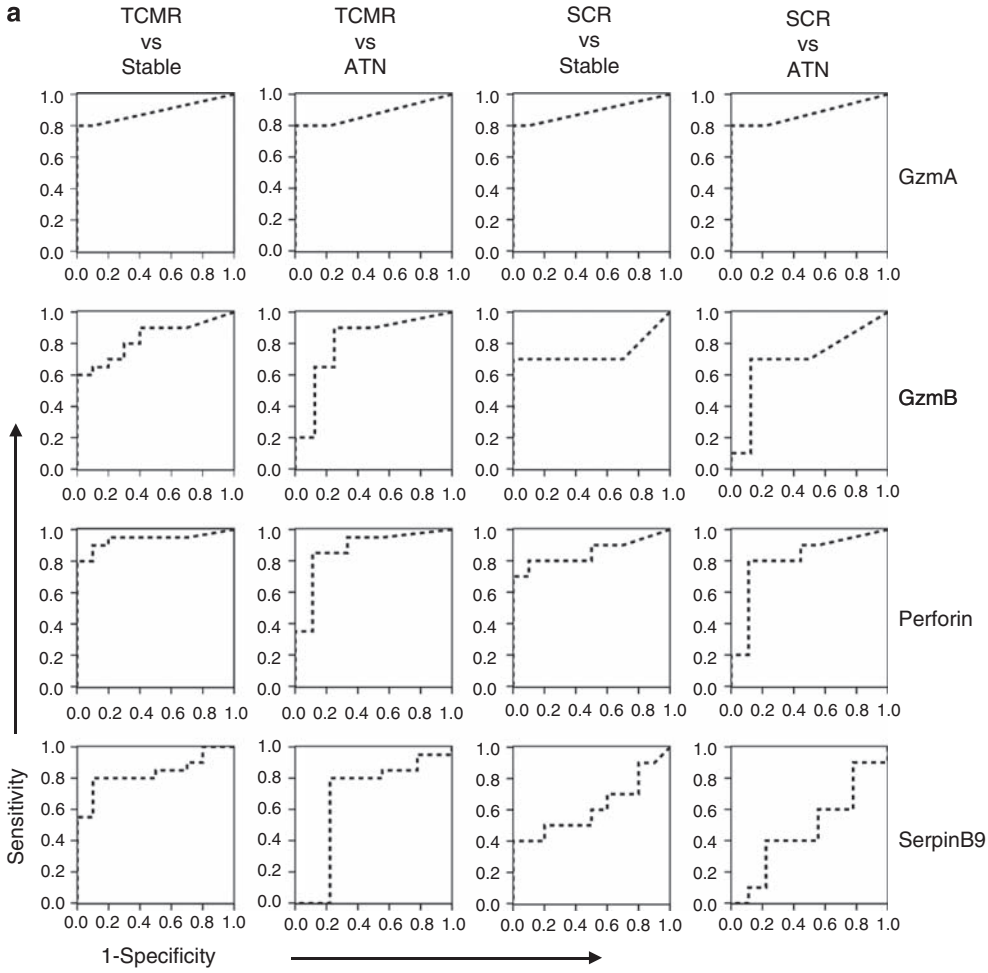
Our data show that urinary GzmA mRNA is a biomarker that can distinguish both SCR and TCMR-I from stable graft function and ATN with good sensitivity and specificity. Comparable gene expression patterns found in SCR and TCMR-I indicate an active ongoing inflammatory process during SCR. In accordance with previous studies,[14–16,22,23] we found that GzmB, perforin, and serpinB9 were significantly elevated during TCMR-I compared with stable grafts. In contrast to GzmA, these markers did not distinguish SCR from stable grafts or ATN.

Cellular infiltration is not invariably associated with immediate graft dysfunction.[24] SCR is a common complication, although reported frequencies vary.[10,11] The strong correlation between SCR and chronic allograft dysfunction[3–6] has persuaded several researches to address the effect of SCR treatment.[7–9] Despite reported beneficial effects on graft histology within 6 months,[7,8] these studies lack to address long-term effects of SCR treatment. SCR is in general disregarded by publications describing non-invasive biomarkers to monitor immunological graft status.[14–16,22,23] Schaub *et al.*[25] showed that urinary CXCL9 and CXCL10 levels correlate with the extent of subclinical tubulitis, suggesting their potential as biomarker. Also other studies have reported urinary biomarkers that are significantly elevated during SCR.[26–28] On the basis of the presented data, urinary GzmA mRNA entails high potential to serve as a non-invasive tool to screen for SCR and to monitor the efficacy of treatment in

Figure 2 | Urinary granzyme A (GzmA) mRNA is a sensitive and highly specific diagnostic biomarker to distinguish subclinical and acute cellular rejection from stable graft function and acute tubular necrosis. (**a**) The graphs represent receiver operating characteristic (ROC) curves visualizing the proportion of true negative results (sensitivity) and true-positive results (specificity) using urinary mRNA levels to discriminate between subclinical rejection (SCR) or T-cell-mediated rejection type I (TCMR-I) from respectively stable or acute tubular necrosis (ATN). An area under the curve value of 0.5 is no better than expected by chance; a value of 1.0 reflects a perfect marker. The upper row of ROC curves represents GzmA mRNA followed downwards by GzmB, perforin, and SerpinB9. GzmA is the most powerful diagnostic biomarker to distinguish both types of rejection from stable and ATN as visualized by a high sensitivity and specificity (see Supplementary Table S4 for accuracy characteristics). (**b**) The value of GzmA and GzmB mRNA levels in differentiating SCR or TCMR-I from stable graft function and ATN is visualized in 2×2 contingency tables. The data represent the number of patients with, respectively, a true-positive, false-positive, true-negative, or false-negative diagnosis of rejection based on urinary mRNA levels, as compared with biopsy findings; the reference standard. The best cut-off points were based on the ROC curve with preference for high specificity.

a patient-convenient manner, provided no CMV infection is present. Correlation between rejection and expression of cytolytic genes in blood[29,30] and urine[14–16] has been addressed by others.[12] Next to GzmB and perforin, urinary serpinB9 mRNA is also elevated during TCMR-I compared with stable grafts.[23] Implementation of these parameters as diagnostic tools is particularly hampered by overlapping expression levels in patients with and without rejection.

GzmA, GzmB, perforin, and serpinB9 are all abundantly expressed in cytotoxic T lymphocytes and natural killer cells, but their expression varies between subsets.[31] Once released into the cytoplasm, granzymes activate caspase-independent



b

GzmB	TCMR	Stable	*P-value*
> 0.00	16	0	*0.001*
≤ 0.00	4	10	

GzmB	TCMR	Stable	*P-value*
≥ 0.01	16	3	*0.004*
< 0.01	4	7	

GzmA	TCMR	ATN	*P-value*
> 0.00	16	2	*0.002*
≤ 0.00	4	7	

GzmB	TCMR	ATN	*P-value*
> 0.00	18	1	*0.013*
≤ 0.00	2	7	

GzmA	SCR	Stable	*P-value*
> 0.00	8	0	*0.007*
≤ 0.00	2	10	

GzmB	SCR	Stable	*P-value*
≥ 0.02	7	0	*0.112*
< 0.02	3	10	

GzmA	SCR	ATN	*P-value*
> 0.00	8	0	*0.008*
≤ 0.00	2	10	

GzmB	SCR	ATN	*P-value*
≥ 0.01	7	0	*0.155*
< 0.01	3	10	

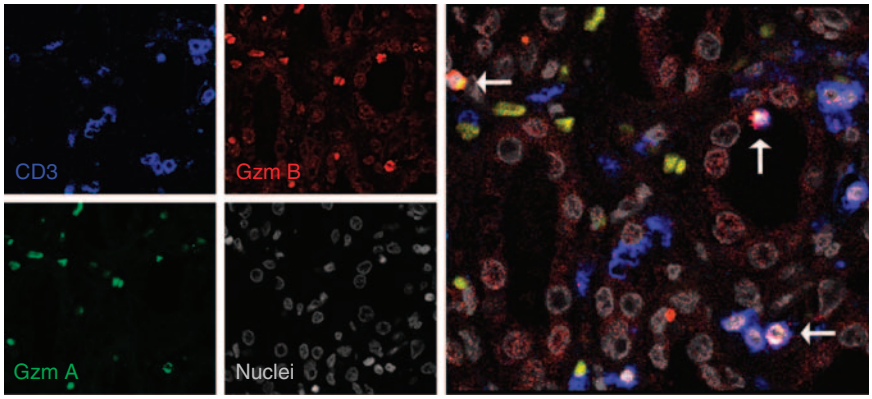


Figure 3 | Expression and colocalization of granzyme A (GzmA), granzyme B (GzmB) by graft infiltrating T cells and natural killer cells during rejection. Triple immunohistochemical stainings were performed to identify the source of GzmA and GzmB during renal allograft rejection. Depicted is a biopsy with TCMR-I showing triple staining with CD3 (blue), GzmA (green), GzmB (red), nuclear counterstaining (gray), and overlay. Many graft infiltrating cells are CD3-positive T cells containing granzymes, for example, the triple positive cells located inside a tubulus and in the interstitium (white arrows). Granzyme-double positive, CD3-negative cells were also present at a lower frequency, for example, the cell at the upper left. The intensity and ratio of GzmA and GzmB differed per cell.

and -dependent cascades eventually leading to target cell death.[17] Emerging evidence shows that the granule secretory pathway may have an unexpected role in inflammation. Secreted GzmA induced proinflammatory cytokines; in this manner GzmA can modulate immune responses.[18,19] GzmB, on the other hand, is the prime inducer of cell death.

On the basis of our data, urinary GzmA did not correlate with creatinine levels during rejection, nor did the other genes tested (data not shown). Furthermore, GzmA expression was not linked to 6 months or 1-year graft survival, severity of rejection, or effect of treatment (data not shown). To test the value of GzmA as diagnostic biomarker, a clinical trial with frequent urine sampling and protocol biopsies is needed.

GzmA mRNA was undetectable in the majority of TCMR-II samples, which was unexpected as both types of TCMR are characterized by graft infiltrating lymphocytes. Our findings do correspond to the lower levels of urinary perforin and GzmB mRNA found in patients with TCMR-II compared with TCMR-I described by Li *et al.*[16] We observed a correlation between measurable GzmA and presence of tubulitis in corresponding biopsies (data not shown). GzmA containing cells were undetectable in urine of TCMR-II with limited tubulitis, suggesting that location of lymphocytes inside the tubule lumen is necessary for urinary detection. Owing to the low frequency of antibody-mediated rejection in our center, we could not address the effect of humoral immune responses on urinary gene expression. As TCMR and antibody-mediated rejection have different treatment strategies, it would be very interesting to know whether GzmA could distinguish between these forms of rejection.

Next to rejection, kidney transplant recipients are commonly challenged with ischemic–reperfusion injury, side effects of immunosuppressive drugs, and CMV infection. In our study, GzmA expression was undetectable in almost all patients with ATN and calcineurin inhibitor toxicity. CMV infection is characterized by high numbers of circulating $GzmB^+$ virus-specific T cells,[32] elevated GzmB, and perforin mRNA levels in blood and increased serum levels of soluble serpinB9.[33] Urinary GzmA mRNA was significantly elevated during CMV infection compared with stable and ATN. However, this is not a limit to the implementation of GzmA as a diagnostic biomarker, as CMV can easily be ruled out by PCR. Moreover, prophylactic antiviral therapy can prevent infection in the early post-transplant period when the majority of rejection episodes occur.[2,4]

In conclusion, urinary GzmA mRNA entails a sensitive and specific diagnostic biomarker to distinguish SCR and TCMR-I from stable grafts and ATN. Routine monitoring of urinary GzmA might be a useful tool to study immunological graft status in a reliable and patient-convenient manner.

MATERIALS AND METHODS

Kidney transplant patients

We included 70 renal transplant recipients between 2004 and 2008; all participants gave written informed consent. All patients received anti-interleukin-2R monoclonal antibodies (Basiliximab), prednisone, calcineurin inhibitor (Tacrolimus or Cyclosporin), and mycophenolate mofetil in standard doses. TCMR was treated with methylprednisolone 500 mg for 6 consecutive days. Antithymocyte globulin was given for steroid-resistant rejection. SCR was not treated in this cohort.

Biopsies were taken at 6 and 12 months after transplantation, upon clinical indication, and according to local standard of practice and scored using the Banff '05 classification.[34] Patients were divided into six groups; normal histology with stable function (stable grafts: $n=10$), TCMR-I ($n=20$) showing prominent tubulitis, TCMR-II ($n=7$) with moderate or severe intimal arteritis and tubulitis and negative for C4D staining, ATN ($n=9$) with no lymphocyte infiltrates, SCR ($n=10$), and calcineurin inhibitor toxicity ($n=4$). SCR was defined as histopathological TCMR without simultaneous decline of graft function; including acute (Banff grade IA or higher, $n=2$) and borderline SCR (tubulitis score 1 and infiltration score 1–2, without arteritis, $n=8$). All patients included were CMV negative, based on CMV-PCR. Additionally, 10 patients who experienced a CMV infection (primary $n=6$ or reactivation

$n = 4$) with measurable viral replication but who remained free of rejection were studied.

Immunohistochemistry

Expression of GzmA, GzmB, and CD3 was analyzed immunohistochemically on paraffin-embedded biopsies (Supplementary Table S1 online). Nuclei were counterstained with TO-PRO3 (Invitrogen, Carlsbad, CA, USA). Triple stainings, GzmA, GzmB, and CD3 were evaluated with a Confocal Laser Scanning Microscope (Leica TCS-SP2, Leica Microsystems B.V., Rijswijk, The Netherlands). Tonsil tissue was used as a positive control.

Urine sample collection

Fresh urine samples ($n = 149$, 100 ml) were collected weekly, up to 10 weeks after transplantation. In case of a biopsy, an extra sample was obtained before treatment. RNA was isolated from the urinary cells with the GenElute Mammalian Total RNA kit (Sigma, Zwijndrecht, The Netherlands) according to manufacturer's instructions.

Semiquantitative real-time mRNA measurements

The isolated RNA was quantified and reverse transcribed to complementary DNA. Primers for GzmA, GzmB, perforin, and serpinB9 were developed to span exon–intron junctions (Supplementary Table S2 online). Primers were validated on complementary DNA of lymphokine-activated killer cells, generated by culturing isolated peripheral bone marrow cells of healthy volunteers with 6000 U/ml interleukin-2 for 7 days. Gene expression was measured in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the SYBR green method (Applied Biosystems). Thresholds of detection were set at the lowest C_t values within the linear amplification range. Transcript levels and relative gene expression of mRNA were determined as described by Pfaffl.[35] All results were normalized to the internal control 18S rRNA, and are expressed relative to the expression in lymphokine-activated killer cells.

Statistical analyses

Patient characteristics are presented as respectively median and quartiles or percentages. Statistical analyses were performed in GraphPad Prism (version 5, GraphPad Software, La Jolla, CA, USA) and SPSS (version 16.0, SPSS, Chicago, IL, USA). A Kruskal–Wallis or χ^2-test was used when appropriate. Experimental data are presented as relative gene expression, median, and quartiles. Multiple two-tailed Mann–Whitney *U* tests were used to compare mRNA levels between groups. To control the false discovery rate, we performed a Benjamini–Hochberg correction; a *P*-value below 0.02 was considered as significant. Receiver operating characteristic curves were used in order to determine cut-off mRNA levels yielding the highest specificity combined with good sensitivity. Correlations between urinary mRNA and clinical parameters were addressed by calculation of Spearman's rank or Pearson's correlation coefficients.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. Primers for granzyme A, granzyme B, perforin, serpinB9, and 18S-rRNA.
Table S2. Antibodies used for immunohistochemical triple stainings of kidney biopsies.
Table S3. Relative Granzyme A, Granzyme B, perforin, and serpinB9 mRNA expression levels in urine of renal transplant recipients.
Table S4. Accuracy characteristics of the tested gene markers for the diagnosis of rejection based on granzyme A, granzyme B, perforin, or serpinB9.
Figure S1. Urinary messenger RNA expression of granzyme A, granzyme B, perforin, and serpinB9 in renal transplant recipients.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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