Non-Invasive Tool to Distinguish Between Acute Rejection and Stable Graft Function Using Urinary mRNA Signature Reflecting Allograft Status in Kidney Transplantation

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Abstract

Background: Urine has been regarded as the best resource based on the assumption that urine can directly reflect the state of the allograft or ongoing injury in kidney transplantation. Previous studies, suggesting the usefulness of urinary mRNA as a biomarker of acute rejection, imply that urinary mRNA mirrors the transcriptional activity of the kidneys.

Methods: We absolutely measured 14 data-driven candidate genes using quantitative PCR without pre-amplification in the cross-sectional specimens collected from Korean kidney transplant patients. We developed a clinical application model adopting urinary mRNAs for allograft rejection using a binary logistic regression and finally verified its usefulness in a large-scale validation group.

Results: We measured the candidate genes in 103 training samples. Expression of 8/14 genes were significantly different between acute rejection and stable graft function with normal pathology and long-term graft survival. Also, CXCL9 was distinctly expressed in allografts with acute rejection in in situ hybridization analysis. This result, consistent with the qPCR result, implies that urinary mRNA could reflect the magnitude of allograft injury. We developed AR prediction model by differently combined mRNAs and the area under the curve (AUC) of the model was 0.89 in training set. The model was validated in 391 independent samples, and the AUC of the model was 0.84 with a fixed manner. In addition, the decision curve analysis indicated a range of reasonable threshold probabilities for biopsy.

Conclusions. Therefore, we suggest urinary mRNA signature may serve as a non-invasive monitoring tool of acute rejection and intragraft immune injury.

Introduction

Kidney transplantation (KTx) provides better quality of life in patients with end-stage renal disease (ESRD), but KTx patients often experience allograft failure due to acute rejection (AR). Clinically applicable immune monitoring is necessary to minimize AR and prevent side effects such as infection and malignancy caused by the use of excess immunosuppressive drugs in kidney transplant recipients [1, 2]. In the past, various tests such as ATP assay, immune cell analysis, and determination of cytokines in blood and urine have been introduced [3–7], but the usefulness of these tools for monitoring kidney graft status is yet to be evaluated in clinical trials.

Urine samples have been considered a good source of factors to monitor allograft status by biomarker researchers in urine of KTx patients [8], because cells contained in urine comprise various molecules that reflect the biological processes of allograft or ongoing kidney injury and because urine can be easily sampled for serial monitoring of the kidney allograft by a truly noninvasive manner in the clinical setting. In a number of studies previously evaluated for urine biomarkers, quantitative real-time PCR (qPCR) has been used for analyzing a few mRNAs that are biologically expected to reflect the immune status of rejection in urine. Other high-throughput approaches such as microarray analysis and RNA sequencing are ideal for the discovery of target genes associated with a specific disease within the whole transcriptome, but it is not easy to profile gene expression in urine samples because of low amounts of total RNA. Thus, we conducted a meta-analysis from public datasets of biopsy transcriptome to select proper candidates for diagnosing AR in KTx patients.

We previously established qPCR assays to measure absolute and relative amounts of urinary mRNAs using 18S rRNA as a reference [9]. In this study, we identified biomarkers for diagnosing AR in urinary cells using the qPCR for absolute quantification without pre-amplification. We developed a signature to distinguish patients with AR from patients with stable graft function (STA). After validation in independent samples (n = 391), and by using decision curve analysis, we evaluated whether the signature is useful for physicians in deciding to perform kidney allograft biopsy.

Materials And Methods

Patients

In Assessment of immunologic Risk and Tolerance in Kidney Transplantation (ARTKT-1) study, which was a cross-sectional sample collection study, we enrolled 543 KTx patients at Kyung Hee University Hospital at Gangdong from 2012 to 2015 and five different hospitals (Kyung Hee Medical Center, Kyungpook National University Hospital, Samsung Medical Center, Seoul St. Mary's Hospital of the Catholic University of Korea, and Inje University Busan Paik Hospital) from 2013 to 2015. We used the Modification of Diet in Renal Disease (MDRD) equation to estimate the glomerular filtration rate (GFR). Indication biopsy was taken upon any clinical indication, but protocol biopsy was performed at 2 weeks (KHG and KMC) or 3 months (CMC) based on the institution's policy, and 457 biopsy-proven samples classified by pathologists at each hospital who were unaware of the analyzed results of the samples using their clinical status and Banff 2007 classification [10] and 86 non-biopsied patients were enrolled.
In 457 biopsy-proven samples, there were normal pathology (NP, n=119), acute T cell-mediated rejection (TCMR, n=75), borderline changes (BC, n=58), acute antibody-mediated rejection (ABMR, n=26), chronic active antibody-mediated rejection (cABMR, n=25), BK virus nephropathy (BKVN, n=15), acute tubular necrosis (ATN, n=30), calcineurin inhibitor toxicity (CNI, n=28), glomerulonephritis (GN, n=27), interstitial fibrosis/tubular atrophy (IF/TA, n=12), and others (n=42). In addition, we enrolled 79 non-biopsied patients defined as long-term graft survival (LTGS) with maintaining stable kidney function while on maintenance immunosuppression for more than 10 years after KT and 7 non-biopsied patients defined as operational tolerance (OT) with maintaining stable kidney function (serum creatinine < 1.5 mg/dl) without any immunosuppressive drugs for more than 1 year. We excluded 42 biopsy-proven samples with acute pyelonephritis, acute TI nephritis, diabetic nephropathy, hydronephrosis, hypertensive glomerulopathy, microvascular injury, non-specific injury, unknown, and 7 OT samples because of small scale, less frequent findings, or unclearly defined specimens for pathological classification in this study.

**Study design and clinical characteristics of patients**

We supplemented LTGS samples for a small number of normal pathology group as STA and split into a training set of AR (n=58) and STA (n=45) groups randomly selected by a clearly defined pathology. We first measured 14 genes in 103 training samples for gene selection by absolute qPCR method without pre-amplification, and then developed AR model by statistical analysis. In the next step, we finally measured 6 genes to verify the discriminative power of the model in 391 independent samples, which consist of STA (n=153), AR (n=68), borderline changes (BC, n=58) for TCMR, BKVN (n=15), and other graft injuries (OGIs, n=97) including acute tubular necrosis (ATN, n=30), calcineurin inhibitor (CNI, n=28) toxicity, glomerulonephritis (GN, n=27), and interstitial fibrosis/tubular atrophy (IF/TA, n=12). We assessed AR prediction model with the fixed cutoff point and evaluated whether the decision curve analysis of the signature is better than biopsy for clinical management and diagnosis of AR. The work-flow chart of this study is shown in Figure 1.

There was no significant difference in the mean age of patients and maintenance immunosuppression between the groups. The duration after KT was significantly longer in STA group than others because this group contained the patients with long-term good graft function, whose follow-up periods after KT were over 10 years. STA group showed lower proportion of deceased donor KT and low HLA mismatching numbers than other groups. Residual graft function was significantly high in STA group compared to others in both training and validation set. The baseline clinical characteristics of the study population are summarized in Table 1.

**Selection for AR candidate genes**

To select AR candidate genes, we searched “kidney rejection” keyword in the whole GEO database. Among the initially queried result, we filtered further by species (Homo Sapience) and data sets using the gene expression platform (Affymetrix GeneChip U133+2) to minimize the unexpected bias by platform difference. We manually examined the top 10 data sets in terms of the sample sizes and excluded sets that do not have acute rejection and stable samples. We found the significantly different genes between STA and AR using GeneMeta R package in four data sets (Supplemental Table 1), following the approach of Choi et al [11]. In total, 137 probe-sets corresponding to 109 unique genes were found to be significant across multiple data sets (FDR<0.01 and FC>2) by false discovery rates (FDRs) were obtained from 1,000 permutations. The genes in the order of FCs were further examined for the clarity of gene annotation, and 10 genes were finally selected (Supplemental Table 2).

In addition, we selected representative genes (CD3e, Foxp3, OX40, and Tim-3) that are well known as diagnostic markers for allograft rejection through literature review (Supplemental Table 3).

**Urine collection and qPCR by absolute quantitation**

Urine samples (50 ml) in each center were collected at the time of biopsy using an identical protocol, except for the patients with long-term graft stable function, which were collected at the time of visiting hospital. After the urine was centrifuged at 2,000x g for 20 min, the pellet was transferred into RNAlater and stored at -80°C until use. The urine pellets were shipped to Kyung Hee University Hospital at Gangdong for the measurement of mRNA. Total RNA was extracted from the urinary pellets using a PureLink RNA Mini Kit (Invitrogen) according to the manufacturer’s recommendations. The quantity (absorbance at 260) and purity (the absorbance at 260/280) of RNA were measured using a NanoDrop® ND-2000 UV spectrophotometer (Thermo Scientific). The median (25th and 75th percentile) quantity (µg) of total RNA was 0.74 (0.250-1.680), and the median (25th and 75th percentile) purity of RNA was 1.85 (0.50-2.01) in 494 total samples. Also, we assessed the quality of urinary RNA using 18S rRNA ≥ 1x10^4 copies/ug and TGF-β1 ≥ 1x10^2 copies/ug as quality control (QC) parameters for the improvement of data quality before the measurement of urinary mRNA levels. In 494 total samples, 402 samples (81%) passed QC (Supplemental Table 4).

Reverse transcription was performed with total RNA using M-MLV RT enzyme (200 U/µl; Mbiotech, Inc., Seoul, Korea), and absolute quantities of the mRNAs were measured by TaqMan probe qPCR assays without pre-amplification step. Each DNA oligo serially diluted from
1x10^{-1} to 1x10^{-8} ng/ul was used for standard curve. Copy number of each mRNA was calculated using the molecular weight of DNA and standard curve. The missing value of mRNA was calculated by replacing 40 C_t value in the blank for data analysis. And then, mRNA values were normalized by 18S rRNA copies (x10^{-6}) used as an endogenous control, and log_{10}-transformed to reduce deviation were used in data analysis [9].

In Situ Hybridization Assays

We performed in situ hybridization (ISH) assay for CXCL9 expression in renal biopsy tissues (3 NP, 3 acute TCMR, and 3 acute ABMR) using the RNAscope 2.5 assay kit (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's recommendations. Briefly, formalin-fixed paraffin embedded sections were subjected to deparaffinization, proteolytic digestion with enzyme denaturation, and hybridization with CXCL9 probe. The RNAscope target was retrieved at 95°C for 15 min and then incubated with RNAscope enzyme for 15 min. The hybridization of the probes for CXCL9, human peptidylprolyl isomerase B (positive control), and diaminopimelate B (negative control) were incubated at 40°C for 2 hours. The preamplifier, signal enhancer, amplier, and label probe were sequentially incubated with the samples at 40°C for 30, 15, 30, and 15 min, respectively. After each step, sections were washed two times, and hybridization signals were detected by 3,3-diaminobenzidine (DAB) staining, followed by counterstaining with Gill's hematoxylin.

Statistical Analysis

We compared the levels of transcripts between the STA and AR groups by the non-parametric Mann-Whitney test and assessed differences among the STA, AR, BKVN, and OGi groups by the non-parametric Kruskal-Wallis test using SPSS for Windows, version 20.0 (IBM Corp, Armonk, NY).

We developed a model to predict AR by a binary logistic regression with all significantly altered genes [12]. To diagnose AR using a model, we used a decision cutoff point maximizing the Youden's index, which is the sum of sensitivity and specificity [13]. Receiver operating characteristic (ROC) curve analyses and decision curve analyses were conducted to evaluate the model [14, 15].

Results

Urinary mRNA Levels in the Training Set and ISH in Biopsy Tissues

We measured the expression levels of 14 genes using absolute qPCR without pre-amplification step in the training set. If mRNA is undetected, the absolute value of the mRNA was calculated with standard curve by replacing 40 C_t value in the blank, and each mRNA level was log_{10}-transformed after normalization with 18S rRNA copies (x10^{-6}). The levels of CXCL9, C1QB, LCK, CD3ε, Foxp3, Tim-3 (P<0.001 for each mRNA), IP-10 (P<0.01), PSMB9 and FAM26F (P<0.05) were significantly elevated in the AR group compared to STA, but those of OX40, IDO1, ISG20, vWF, and PTPRC were no difference (Figure 2a and Supplemental Table 5). The copy number of 18S rRNA per total RNA amount was no difference between AR and STA group. Although LCK, Foxp3, and FAM26F mRNAs were statistically significant, these mRNAs were missing values in 14% (n=12), 26% (n=22), and 58% (n=49) of the QC-passed samples (n=84), respectively. Thus, we excluded these mRNAs (FAM26F, Foxp3, and LCK) with low detectable frequency and (OX40, IDO1, ISG20, vWF, and PTPRC) with no statistical difference between AR and STA for further analysis. In addition, we investigated representatively CXCL9 level using in situ hybridization (ISH) analysis to confirm whether mRNA level in kidney biopsy tissue is consistent with the result of urinary mRNA by qPCR assay. CXCL9 was distinctly expressed in the damaged tubules in kidney allografts of acute TCMR and predominantly in peritubular capillary area in ABMR groups, consistent with the results of qPCR analysis (Figure 2b).

Development of Prediction Signature to Distinguish AR from STA

We performed ROC curve analysis to evaluate each target for AR diagnosis, and the AUC values of individual gene were not sufficient to distinguish AR from STA (Supplemental Table 6). Therefore, to improve diagnostic accuracy of AR, we developed AR prediction model using a binary logistic regression in the training set. The level of each mRNA was log_{10}-transformed after each mRNA copy number was normalized with 18S rRNA (x10^{-6}). The AUC value of the signature was 0.89 (95% CI, 0.82-0.96; P<0.001) (Figure 3a). The equation of a six-gene signature is shown below:

\[ \ln \frac{p(x)}{1-p(x)} = -6.582 + (0.404 \times \text{C1QB}) + (0.998 \times \text{CD3e}) + (2.206 \times \text{CXCL9}) + \\
(-0.904 \times \text{IP10}) + (-1.829 \times \text{PSMB9}) + (2.207 \times \text{Tim3}) \]
With the cutoff point (0.40889), the predicted probability of the signature also yielded 86% accuracy, 91% sensitivity, 80% specificity, 83% positive predictive value (PPV), and 89% negative predictive value (NPV) (Figure 3b and Table 2). The signature improved the diagnosis of AR from STA compared to Tim-3 alone.

**Validation of the Diagnostic Signature to Predict AR**

We measured six genes in 391 independent samples including STA, AR, BC, BKVN and OGI groups and evaluated the discriminative power of the diagnostic signature. All of 6 genes were significantly increased in BKVN group as well as AR group compared to STA by Kruskal-Wallis nonparametric test and Dunn’s post-hoc test for multiple comparisons. Interestingly, the expressions of CXCL9 and IP-10 genes (P<0.01) in BKVN were significant higher rather than those in AR group. Scatter plot of the mRNAs and the comparisons between groups by Mann-Whitney t test are shown in Supplemental Figure 1 and Table 7.

To evaluate the performance of the AR predicted signature with the fixed cut-off point, we tested its performance in AR (n=57) and STA (n=122) in the QC-passed samples. The AUC value of the signature yielded 0.84 (95% CI, 0.78-0.90; P<0.001) (Figure 4a) and the predicted probability of the signature showed well discrimination of AR from STA, but it was difficult to discriminate BC (n=42) from AR or STA in validation set (Figure 4b). With the fixed cutoff point, the signature displayed 70% sensitivity (95% CI, 57%-82%) and 80% specificity (95% CI, 72%-87%). Also, overall performance of the signature yielded 77% accuracy, 63% PPV, and 85% NPV (Table 3). Furthermore, we assessed the potential of the signature for distinguishing AR from no-AR group (STA and OGI, n=207) in the QC-passed samples excluding BC and BKVN groups. The signature yielded AUC value of 0.78 (95% CI, 0.72-0.85; P<0.001) with 70% sensitivity (95% CI, 57%-82%), 72% specificity (95% CI, 65%-78%), 77% accuracy, 63% PPV, and 85% NPV (Figure 4c-d and Table 3).

**Decision Curve Analysis of the Diagnostic Signature**

Using decision curve analysis, we assessed whether the analysis of the signature was better than biopsy for clinical management and diagnosis of AR (Figure 5). Based on the decision curve analysis, the range of reasonable threshold probabilities (\(p_t\)) with the highest net benefit was from 0.2 to 0.5 for the diagnostic signature. Ultimately, the diagnostic signature within a reasonable threshold probability was better than biopsy for diagnosing AR after kidney transplantation.

**Discussion**

The expression of AR-specific mRNAs in urinary cell pellets for monitoring the immune status of kidney transplant patients have been studied by many investigators in the last decade. Absolute quantification with preamplification, which was developed by Suthanthiran et al., has been regarded as the standard method for biomarker research using urinary mRNA quantification in kidney transplantation [16]. Although preamplification is reported to be effective in the detection of mRNAs with very low amount, there have been still several concerns regarding the clinical application of preamplification assisted qPCR, such as inconsistent preamplification efficiency, reproducibility, and specificity [17-19]. In this study, we conducted qPCR in the absence of preamplification process and showed that more than 80% of target mRNAs (11/14) were stably measured in most patients of training set. However, missing data of Foxp3, FAM26F, and vWF were 26%, 58%, and 60%, respectively in in training set. Also, LCK, Foxp3, and FAM26F were statistically significant, but we did not rigorously use to further analyze the mRNAs with more than 10% missing data. Some genes with large missing data may be due to the reason without preamplification. Foxp3 mRNA has been suggested as a noninvasive biomarker for AR, but a study recently reported that Foxp3 mRNA was undetectable in most of patients with acute tubular injury [20]. We also observed similar result in most of the samples and thus did not exclude Foxp3 mRNA in data analysis. [21, 22]. Even so, we suggest that qPCR assay without preamplification be an available method for the measurement of AR-specific urinary mRNAs.

We performed a meta-analysis with GEO data sets on biopsy samples from patients with AR and STA to search reasonable molecules and identified 10 genes. Moreover, in gene set enrichment analysis, most of pathways in total were related to immune signaling pathway, and 9 genes among 10 candidates identified by meta-analysis were associated to inflammatory response and immune system (Supplemental Figure 2). We also selected an additional 4 genes (CD3e, Foxp3, OX40, and Tim-3), because urinary mRNA studies suggested that the expression level of CD3e, Foxp3, OX40 or Tim-3 mRNA was higher in patients with AR than in patients with STA, and thus the measurement of these mRNAs may be a promising noninvasive tool for the diagnosis and prediction of AR [16, 23-25]. Additionally, in our ISH study, CXCL9 was distinctly expressed in the damaged tubules in kidney allografts of acute TCMR and predominantly in peritubular capillary area in ABMR groups, consistent with the results of qPCR analysis, but not in those of the STA group. Thus, we showed that the result using ISH analysis was identical to the result obtained by qPCR analysis and that cells in urine could well reflect ongoing kidney injuries or kidney allograft status.
Indeed, in most previous studies that investigated novel biomarkers for rejection, molecular signatures were not locked down while they were examined in validation set [26]. In this study, the diagnostic signature was importantly fixed without any further modification and showed good discrimination potential with AUC of 0.84 in independent validation, suggesting that it is highly likely to be reproducible in clinical practice. The performance of the signature in distinguishing AR from No-AR in the validation set was also acceptable with AUC of 0.78. However, the signature was limited in distinguishing AR from BC and BKVN because all six genes were also significantly elevated in the BC and BKVN group compared to STA, similar to rejection. Furthermore, in decision curve analysis to assess the performance of the signature for clinical benefit, the diagnostic signature had the highest net benefit than biopsy at a \( p \) of 0.2. Therefore, our signature is low in the relative harms and costs compared to kidney biopsy for patients.

BK virus is a major causative agent of nephropathy and can lead to deterioration of the transplanted kidney and graft failure [27]. Several biomarkers have been proposed for the diagnosis of BKVN, such as heat shock protein 90-\( \alpha \) [28], CXCL9 [29], neutrophil gelatinase-associated lipocalin [30], and BK virus-specific CD4 T cells [31]; however, none of the biomarkers have demonstrated superiority over viral DNA test in blood and urine. These results are explained by the fact that a substantial proportion of patients with BKVN also have AR or that the mechanism of immunological graft injury in BKVN might be similar to that in AR [32]. Fortunately, BK virus DNA tests in blood and urine are effective for differentiating BKVN from AR in the clinic. In addition, a more sensitive and specific method for BK virus detection including the presence of viral-specific miRNA has been introduced [33].

There are several limitations in this study. The signature requires six-gene specific standard curves and is limited in clinical practice because it will need additional work. We designed AR and STA groups in training set and OGIs group as well as AR and STA in validation set. It would be that the AUC was reduced due to the reason in validation including OGIs group. Furthermore, it would be the critical limitation that about 20% of urine samples did not pass quality-control in clinical practice. However, the signature successfully distinguished acute rejection from stable and No-AR.

**Conclusion**

In conclusion, we performed a meta-analysis to discover more proper biomarkers for the diagnosis of AR using cells in urine and validated the signature to diagnose and predict AR. Furthermore, using ISH analysis, we showed that cells in urine could well reflect ongoing kidney injuries or kidney allograft status. Therefore, our results demonstrate that the signature can be a noninvasive tool to assist with deciding whether to perform a biopsy in a recipient with a rise in creatinine and probably justifies a biopsy. However, it is further necessary to evaluate the performance of the signature for clinical usefulness in a prospective cohort.

**Declarations**

**Ethics approval and consent to participate**

At the time of transplantation, none of the transplant donors were from a vulnerable population and all donors or next of kin provided written informed consent that was freely given. All kidney transplant patients provided written informed consent to participate in the study, and the study was approved by the local institutional review board at Kyung Hee University Hospital at Gangdong (#2012-030, KHNMC) and registered with the Clinical Research Information Service (KCT0001010).

**Consent for publication**

Not applicable.

**Availability of data and materials**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Author's contributions**
S-H.L. participated in conception and design of the study; J-W.S. and S.H.P. carried out experiments; J-W.S., J-Y.M., K.H.J., C-D.K., B.H.C., J.B.P., S.H.J., S.H.L., and Y.H.K. participated in acquisition of data; J-W.S., D.H.T., and Y-H.L. participated in analysis and interpretation of the data; J-W.S., D.H.T., and S.H.P. made the figures; J-W.S., J.S., and S-H.L. drafted and revised the paper; all authors approved the final version of the manuscript.

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### Tables

**Table 1. Baseline characteristics and clinical parameters of enrolled patients by study group**
|                                | Training set (n=103) | Validation set (n=391) |
|--------------------------------|----------------------|------------------------|
|                                | STA^                | AR^                    | P value†    | STA^             | AR^              | Borderline changes | BKVN | OGIs‡ | P value‡ |
| Number of patients (n)         | 45                  | 58                     | -           | 153             | 68               | 58                  | 15       | 97     | -        |
| Age (years)                    | 49.7±11.5           | 47.2±10.9              | 0.263       | 48.7±12.0       | 46.7±10.6        | 0.263               | 48.3±11.5 | 47.7±15.7 | 47.7±11.8 | 0.823 |
| Sex (Male, %)                  | 17 (37.8)           | 35 (60.3)              | 0.023       | 98 (64.1)       | 47 (69.1)        | 0.023               | 45 (77.6) | 11 (73.3) | 65 (67.0) | 0.430 |
| Duration after KT (Months)     | 92.2±102.0          | 28.8±43.7              | <0.001      | 73.6±98.5       | 40.2±48.8        | 0.013               | 7.9±15.1  | 11.7±10.5 | 46.5±64.4 | <0.001 |
| Deceased donor KT (n, %)       | 14 (31.1)           | 23 (39.7)              | 0.370       | 47 (30.7)       | 31 (45.6)        | 0.370               | 42 (43.3) | 5 (33.3) | 42 (43.3) | 0.009 |
| ABO incompatible KT (n, %)      | 4 (8.9)             | 13 (22.4)              | 0.068       | 21 (13.7)       | 10 (14.7)        | 0.068               | 6 (10.3)  | 2 (13.3) | 3 (3.1)  | 0.092 |
| HLA mismatching (n)            | 3.0±1.9             | 3.5±1.5                | 0.127       | 3.1±1.5         | 3.7±1.8          | 0.127               | 3.7±1.4   | 3.8±1.7 | 3.2±1.4  | 0.012 |
| Induction immunosuppression     |                      |                        |             |                 |                   |                     |          |        |          |
| Basiliximab (n, %)             | 34 (75.6)           | 39 (67.2)              | 0.357       | 105 (68.6)      | 52 (65.5)        | 0.357               | 38 (65.5) | 10 (66.7) | 73 (75.3) | 0.466 |
| Anti-thymocyte globulin (n, %)  | 11 (24.4)           | 19 (32.8)              | 0.174       | 48 (31.4)       | 16 (23.5)        | 0.174               | 20 (34.5) | 5 (33.3) | 24 (24.7) |          |
| Maintenance immunosuppression   |                      |                        |             |                 |                   |                     |          |        |          |        |
| Steroid (n, %)                 | 32 (71.1)           | 55 (94.8)              | 0.001       | 135 (88.2)      | 56 (82.4)        | 0.001               | 52 (90.7) | 14 (93.3) | 88 (90.7) | 0.504 |
| Tacrolimus (n, %)              | 29 (64.4)           | 38 (65.5)              | 0.910       | 122 (79.7)      | 50 (73.5)        | 0.910               | 54 (93.1) | 14 (93.3) | 80 (82.5) | 0.042 |
| Cyclosporine (n, %)            | 13 (26.7)           | 17 (29.3)              | 0.767       | 26 (17.0)       | 11 (16.2)        | 0.767               | 3 (5.2)   | 0 (0)   | 13 (13.4) | 0.031 |
| Mycophenolate mofetil (n, %)    | 32 (71.1)           | 48 (82.3)              | 0.159       | 134 (87.6)      | 50 (73.5)        | 0.159               | 52 (89.7) | 10 (66.7) | 82 (84.5) | 0.020 |
| mTOR inhibitor (n, %)           | 5 (11.1)            | 3 (5.2)                | 0.266       | 5 (3.3)         | 8 (11.8)         | 0.266               | 3 (5.2)   | 1 (6.7)  | 3 (3.1)  | 0.619 |
| eGFR (ml/min/1.73m²)           | 77.8±17.7           | 36.8±21.0              | <0.001      | 72.3±20.8       | 36.0±18.5        | <0.001              | 53.0±18.5 | 32.1±10.5 | 40.7±22.3 | <0.001 |
| Donor information              |                      |                        |             |                 |                   |                     |          |        |          |
| Age (years)                    | 39.9±13.6           | 48.5±11.8              | 0.001       | 43.3±13.8       | 44.7±14.2        | 0.001               | 49.8±12.0 | 54.1±8.3 | 44.3±13.4 | <0.001 |
| Sex (Male, %)                  | 28 (62.2)           | 34 (58.6)              | 0.711       | 76 (49.7)       | 35 (51.5)        | 0.711               | 33 (56.9) | 6 (40.0) | 48 (49.5) | 0.709 |

Data are expressed as mean±standard deviation or number (percentage).

Abbreviations: STA, stable allograft function; AR, acute rejection; BKVN, BK virus associated nephropathy; OGIs, other graft injuries; KT, kidney transplantation; HLA, human leukocyte antigen; eGFR, estimated glomerular filtration rate; mTOR, mammalian target of rapamycin

^ STA group in discovery set consisted of normal pathology (n=23) and long-term graft survival (n=22).

^ AR group in discovery set consisted of acute T cell-mediated rejection (n=37), and acute antibody-mediated rejection (n=21).

^ STA group in validation set consisted of normal pathology (n=96) and long-term graft survival (n=57).
AR group in validation set consisted of acute T cell-mediated rejection (n=38), acute antibody-mediated rejection (n=11), and chronic active antibody-mediated rejection (n=19).

OGIs include acute tubular necrosis (n=30), calcineurin inhibitor toxicity (n=28), glomerulonephritis (n=27), and interstitial fibrosis/tubular atrophy (n=12).

† For non-normally distributed variables, data were analyzed using the non-parametric Mann-Whitney test.

‡ For non-normally distributed variables, data were analyzed using the Kruskal-Wallis test.

Table 2. Prediction performance of the signature between AR and STA in training set

| Distinction | AUC (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) | Accuracy (95% CI) | Cut-off point | P value |
|-------------|--------------|----------------------|----------------------|--------------|--------------|-------------------|--------------|---------|
| AR vs STA   | 0.89         | 90.91 (78.33% - 97.47%) | 80.00 (64.35% - 90.95%) | 83.33 (72.76% - 90.35%) | 88.89 (75.63% - 95.38%) | 85.71 (76.38% - 92.39%) | 0.408887 | <0.0001 |

PPV, positive predictive value; NPV, negative predictive value

Table 3. Prediction performance of the signature in validation set by the fixed cut-off point

| Distinction | AUC (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) | Accuracy (95% CI) | P value |
|-------------|--------------|----------------------|----------------------|--------------|--------------|-------------------|---------|
| AR vs STA   (n = 179) | 0.84         | 70.18 (56.60% - 81.57%) | 80.33 (72.16% - 86.97%) | 62.5 (52.85% - 71.25%) | 85.22 (79.31% - 89.66%) | 77 (70.24% - 83.03%) | <0.0001 |
| AR vs STA + OGIs (n = 264) | 0.783*       | 70.18 (56.60% - 81.57%) | 71.98 (65.33% - 77.98%) | 40.82 (34.35% - 47.62%) | 89.76 (85.37% - 92.94%) | 72 (65.74% - 76.95%) | <0.0001 |

*AUC was calculated based on AR predicted probability of the signature for patients including AR (n=57), STA (n=122), and no-AR (STA and OGIs, n=207) in QC-passed samples. PPV, positive predictive value; NPV, negative predictive value.