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Immune monitoring of prevalent kidney transplant recipients using Torque Teno Virus: Protocol for a single-centre prospective cohort study

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ABSTRACT

Introduction Kidney transplant recipients (KTRs) suffer from immunosuppression-related adverse events (iRAEs), such as infections and malignancy from chronic immunosuppression, but are also at risk of graft loss from rejection with underimmunosuppression. Biomarkers that predict both iRAEs and rejection while allowing individualisation of immunosuppression exposure are lacking. Although plasma viral DNA levels of torque teno virus (TTV), a widely prevalent, non-pathogenic virus, have been shown to predict both iRAE and rejection in newly transplanted KTRs within the first year after transplant, its role for prevalent KTRs on stable immunosuppression is less clear.

This study aims to determine the prognostic value of TTV levels for severe infections (defined as infections requiring hospitalisation) in prevalent KTRs on stable immunosuppression for at least 3 months and compare it against that of other commonly available biomarkers. The study also aims to explore the relationship between TTV levels and factors affecting the 'net state of immunosuppression' as well as other clinical outcomes.

Methods and analysis This is a single-centre, prospective, observational cohort study of 172 KTRs on stable immunosuppression for more than 3 months. TTV levels will be measured using the TTV R-GENE kit upon recruitment when study subjects are admitted and when kidney allograft biopsies are performed. Subjects will be monitored for iRAEs and rejection for at least 12 months. The relationship between TTV load and clinical outcomes such as severe infections will be analysed and compared against that from other common biomarkers and previously published predictive scores.

Ethics and dissemination The study was approved by the SingHealth Centralised Institutional Review Board (2023/2170). The results will be presented at conferences and submitted for publication in peer-reviewed journals.

Trial registration number NCT05836636.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ While most studies have included newly transplanted patients within the first year after transplant, this study will evaluate the prognostic value of torque teno virus (TTV) for infections and other complications in prevalent kidney transplant recipients who are on stable immunosuppression beyond the immediate post-transplant period.
⇒ The study will also compare the prognostic performance of TTV with other commonly available biomarkers and previously published predictive scores.
⇒ As a single-centre study, generalisability of results to broader populations may be limited.

INTRODUCTION

Kidney transplant recipients (KTRs) are not only at increased risk of immunosuppression-related adverse events (iRAEs), such as infections, malignancy and metabolic complications from chronic immunosuppression exposure, but are also at risk of graft loss from rejection with underimmunosuppression.[1–4] Within our KTR cohort, infections accounted for 41.4% of all deaths and 35.7% of all hospital admissions.[1] Annual rate of hospitalisation due to infections was 18.7%. Similar to other KTR cohorts, malignancy accounted for 7.3% of deaths in our cohort with a standardised incidence ratio of 3.36.[1 2] On the other hand, rejection accounted for a significant proportion (20.1%) of graft loss.[1]

Currently, there is no biomarker that can predict both iRAEs and rejection effectively while allowing individualisation of immunosuppression exposure.[5 6] Peripheral lymphocyte subpopulations, complement levels and immunoglobulin levels may be associated with risk of infection but have not been shown to be associated with rejection.[7–9] The association between infection and rejection with intracellular ATP levels after T cell stimulation was inconsistent.[10] Other functional T cell assays are promising for predicting specific infections such as cytomegalovirus (CMV) reactivation but have not been shown to correlate with rejection.[11] The clinical utility of some of these tests may be limited by

the need for specialised laboratories, the need for specific human leucocyte antigen (HLA) types and high cost.[12] Predictive scores comprising of several demographic and laboratory factors have also been tested but have not been shown to be effective across multiple time points and may be cumbersome to monitor. Moreover, most factors included, such as age and kidney function, may not be modifiable in clinical practice.[13 14] Importantly, most strategies previously investigated may only account for an isolated aspect of the immune system.

Monitoring of donor-specific antibodies (DSAs) and/or donor-derived cell-free DNA (ddcfDNA) has been proposed.[15 16] However, these strategies may be suboptimal since the development of de novo DSAs signifies an immunological event and is associated with poorer outcomes, while positive ddcfDNA already represents significant tissue injury. Protocol biopsies have previously been shown to detect subclinical tissue injury and may potentially improve outcomes, but are associated with complications.[17 18] While short-term outcomes for KTRs have improved significantly, improvements for long-term outcomes have been modest.[19] Therefore, a biomarker to measure the 'net state of immunosuppression' to allow reduction of immunosuppression without an increase in rejection may help improve long-term outcomes.

Torque teno virus (TTV) is a widely prevalent virus that is not known to cause any disease.[20 21] Plasma TTV DNA levels have been shown to correlate with the degree of immunosuppression in solid organ transplant recipients and remain stable over time in patients on stable immunosuppression. Higher TTV levels have been shown to predict infections and other iRAEs, while lower TTV levels can predict rejection.[22–25] Along with other viral kinetic parameters such as the trends and total viral load over time, TTV levels have been shown to predict both iRAE and rejection.[22–32 33]

Most of the currently available data have focused on newly transplanted patients within the first year of transplant.[22] Similarly, an ongoing randomised controlled trial comparing TTV-guided tacrolimus titration against standard of care will only include transplant patients within the first year of kidney transplant.[33] However, the risk of infections and other complications, such as rejection, differs between newly transplanted patients and prevalent KTRs who have been stable or transplanted more than 1 year ago.[5 34] Therefore, the prognostic value and optimal cut-off values of TTV may differ between these two groups of patients.

To our knowledge, only one study by Gore *et al*[35] has examined TTV levels in prevalent KTRs for mortality and death due to an infectious cause beyond the first year of transplant.[22 35] However, data on study outcomes may be incomplete since they were obtained by retrieving data based on diagnostic codes. Important outcomes such as rejection, opportunistic infections and hospitalisation due to infections could not be studied. The accuracy of TTV DNA quantification was also unclear since stored blood samples were used, and samples needed to be diluted due to low sample volumes. The stability and trends of TTV were also unclear as there was only one specimen for each patient. Despite these limitations, the study demonstrated that high TTV levels were associated with all-cause mortality and death due to an infectious cause.[35] Numerous studies have demonstrated that TTV viral loads can predict SARS-CoV-2 vaccine response in prevalent solid organ transplant recipients, providing further evidence that TTV may be a useful indicator of the depth of immunosuppression in prevalent KTRs.[36–40]

No previous studies, to the best of our knowledge, have compared TTV with other biomarkers or predictive scoring systems. It is also unclear if the prognostic performance of TTV levels can be improved by combining it with clinical data, laboratory findings or other biomarkers.

Study objectives

The study aims to determine the prognostic value of plasma TTV DNA levels for severe infections (defined as any infection requiring hospitalisation) and other iRAEs, such as opportunistic infections and malignancy, in our KTRs on stable immunosuppression for more than 3 months—including patients beyond the first year of transplantation. Other outcomes monitored will include biopsy-proven rejection, calcineurin inhibitor toxicity and recurrent or de novo glomerulonephritis.

The prognostic performance of TTV DNA levels will also be compared against other clinical markers such as absolute lymphocyte counts, lymphocyte subpopulation counts (CD4, CD8, CD19, CD56), IgG and C3 levels. The study also aims to compare the prognostic value of TTV against other common biomarkers and two predictive scoring systems by Fernandez-Ruiz *et al* and Dendle *et al*.[13 14]

The study also aims to determine the distribution of TTV DNA levels in our local cohort of KTRs and its relationship with clinical factors affecting the 'net state of immunosuppression' in prevalent KTRs such as age, comorbidities, kidney function and immunosuppression drug levels. Therefore, the results of this study will help inform if TTV may be useful in stable post-transplant patients, including those beyond the first year of transplant, and may allow a more widespread adoption of this promising biomarker.

METHODS AND ANALYSIS

Study outline

This is a single-centre, prospective, observational cohort study (figure 1) which will recruit 172 KTRs on stable doses of immunosuppression for more than 3 months and follow them up for at least 12 months. Plasma TTV DNA levels will be measured upon recruitment together with routine full blood count, creatinine, lymphocyte subpopulation counts (CD4, CD8, CD19, CD56), IgG and C3 levels. Additional TTV DNA levels will be measured when study participants are hospitalised or undergo kidney allograft biopsies (table 1). The primary outcome will

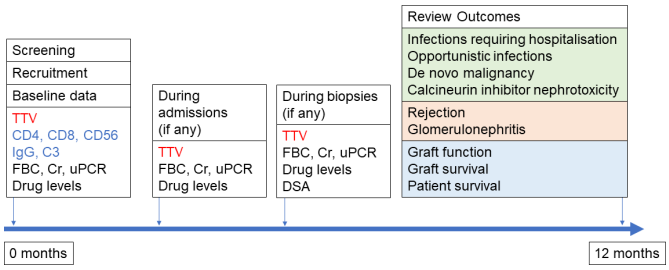

Figure 1 Overview of study design. Cr, creatinine; DSA, donor-specific antibody; FBC, full blood count; TTV, torque teno virus.

be severe infections, defined as any infection requiring hospitalisation.[13]

Study population

Inclusion criteria

All adult (21 years old or older) KTRs on stable doses of immunosuppression for more than 3 months are eligible for the study.

Exclusion criteria

A patient will be excluded from the study if any of the following criteria are met:

- Titration of immunosuppression (eg, for rejection or infection) less than 3 months ago.
- Any clinical event within the past 3 months which may significantly influence the patient's net state of immunosuppression (eg, uncontrolled infections, newly diagnosed malignancy).

Clinical assessment

Baseline data including age, gender, ethnicity, weight, height, history of diabetes mellitus, liver cirrhosis, rejection, infection, malignancy, and transplant data including date and type of transplant, previous transplants, ABO compatibility, HLA compatibility, crossmatch, DSA results, donor and recipient CMV sero-status, induction immunosuppression, and maintenance immunosuppression will be collected for all subjects.

Laboratory data including white cell count, absolute neutrophil count, absolute lymphocyte count, immunosuppression drug level, liver panel, serum creatinine level estimated glomerular filtration rate and urine protein-to-creatinine ratio will be collected upon recruitment. Peripheral lymphocyte subpopulations for CD4, CD8, CD19 and CD56 will be quantified via flow cytometry (BD Multitest, FACSCanto and FACSCanto II), and serum IgG and C3 will be measured upon recruitment. Full blood count, serum creatinine level and estimated glomerular filtration rate, urine protein-to-creatinine ratio and immunosuppression drug level will be obtained when patients are hospitalised or require kidney allograft biopsies. All haematological and biochemistry laboratory investigations will be performed in the central laboratory which is accredited by the College of American Pathologists. Additionally, DSAs will be obtained when patients require kidney allograft biopsies.

Outcomes will be determined by reviewing the electronic medical records of the study participants.

Table 1 Study schedule

Case report forms (CRFs)	0 month	12 months
CRF1—screening	☑	
CRF2—baseline data	☑	
Measurement—TTV DNA level (baseline)	☑	
Measurement—CD4, CD8, CD19, CD56, IgG, C3	☑	
CRF3—review for outcomes		☑
CRF4—biopsy or admission	Ad hoc	
Measurement—TTV DNA level (biopsy or admission)	Ad hoc	

TTV, torque teno virus.

TTV DNA level measurement

One venous blood sample (3 mL) in an EDTA tube for TTV DNA level will be obtained from all study subjects upon recruitment and when subjects are hospitalised or undergo kidney allograft biopsies. Blood samples will be de-identified and sent to the local institutional molecular laboratory. Blood samples will be centrifuged within 24 hours of collection, and plasma specimens will be preserved at −80°C. Genomic DNA will be extracted from 200 µL of blood plasma samples using the EMAG platform (bioMérieux), according to the manufacturer's protocol. DNA loads will be quantified by the TTV R-GENE kit (ARGENE range, bioMérieux), a real-time quantitative PCR (qPCR) assay targeting a highly conserved segment of the 5' untranslated region of the viral genome with >90% identity across isolates.[41] The qPCR reactions will be performed in a volume of 25 µL containing 10 µL of extracted DNA, and primers and probe according to manufacturer's instructions. Thermal cycling will be started for 15 min at 95°C, followed by 45 cycles PCR amplification at 95°C for 10 s, and at 60°C for 40 s, using the CFX96 Real-time System (Bio-Rad, Hercules, California, USA). The viral load (in copy numbers/mL) will be determined using a standard curve with known copy numbers and $\log_{10}$-transformed for statistical analyses.

Outcomes

The primary outcome will be severe infections, defined as any infection requiring hospitalisation.[13]

Secondary outcomes

1. Opportunistic infections including but not limited to the following pathogens: intracellular bacteria, mycobacteria, *Listeria monocytogenes* and *Nocardia* spp, herpesviruses (CMV, herpes simplex virus and varicella zoster virus), polyomaviruses, yeasts (*Candida* and *Cryptococcus*), moulds (invasive aspergillosis and mucormycosis) and parasites (*Toxoplasma gondii*, *Pneumocystis jirovecii* and *Leishmania*).[27]
2. De novo malignancy.
3. Calcineurin inhibitor nephrotoxicity (biopsy proven).

4. Rejection (biopsy proven, with and without borderline T cell-mediated rejection).
5. Glomerulonephritis—de novo or recurrent (biopsy proven).
6. Graft function (serum creatinine, estimated glomerular filtration rate by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation and urine protein-to-creatinine ratio).
7. Graft loss (censored and non-censored for death).
8. Mortality (all-cause and cause specific—that is, infection, malignancy, cardiovascular, others).
9. iRAE—composite outcome of primary endpoint and secondary endpoints (1), (2) and (3).
10. Immune-mediated adverse event—composite outcome of secondary endpoints (4) and (5).

All patients will be followed for at least 12 months, until graft loss (defined as return to dialysis or retransplantation) or death, whichever occurs earlier. The subjects will be reviewed at least every 4 months at the study site. At the end of follow-up, two study team members will independently review the subjects' electronic medical records for the outcomes of interest. A third study team member will arbitrate any disagreements. The study team will be blinded to the subjects' TTV levels.

Statistical analysis

Descriptive statistics will be used to describe the baseline demographics for the cohort, for patients who develop outcomes of interest (eg, severe infections) and those who do not. Withdrawn patients will also be described fully. Results will be expressed as mean and SD for parametric continuous data, median and IQR (25th, 75th percentile) for non-parametric continuous data and as frequency and percentage for categorical data. Log transformation will be performed for non-parametric continuous data (eg, TTV viral load). TTV viral loads will also be categorised into <4.6 $\log_{10}$, 4.6–6.6 $\log_{10}$ and >6.6 $\log_{10}$ copies/mL; ≤3.6 $\log_{10}$ and >3.6 $\log_{10}$ copies/mL (cut-off values based on previous published studies)[22 29 33 42]; and tertiles, quartiles and quintiles.[22]

For continuous variables, the t-test and unadjusted or multivariable linear models, adjusted for important factors, will used to compare groups of patients with and without outcomes of interest. The Mann-Whitney U test was used to compare non-parametric continuous variables. For categorical variables, Pearson's X^2 test, Fisher's exact test and unadjusted or multivariable logistic models, adjusted for important factors, will used to compare groups of patients with and without outcomes of interest.

Receiver operating characteristics (ROC) curves will be plotted for baseline TTV viral loads and outcomes of interest. Area under ROC curves (AUCs) will be calculated. The outcomes of interest will also be analysed using survival analysis techniques. The log-rank test will be used to test for statistical significance. Cox proportional hazards models, both unadjusted and adjusted for important factors, will be used to analyse the relationship between TTV viral load with time to outcomes of interest. Patients who have been withdrawn or lost to follow-up will be censored at their last known observation.

An interim analysis will be performed when all recruited patients have completed at least 3 months of follow-up.

Sample size was calculated based on a precision-based approach. The calculated sample size is 172 subjects (25 in hospitalisation and 147 in non-hospitalisation group) based on the following parameters: expected AUC of 0.75 with a margin of error of ±0.125 (ie, expected AUC would lie between 0.627 and 0.873 with a two-sided 95% CI), 1:6 allocation ratio and 10% loss to follow-up.

Patient and public involvement

None.

Protocol and registration

This study is registered with ClinicalTrials.gov (NCT05836636).

Data management and oversight

Study team members will be responsible for the conduct of the study. Study team members will monitor the data by reviewing a random sample of 10% of completed data. Monitoring will ensure protocol compliance, proper study management and timely completion of study procedures.

Data will be stored on institutional network drives with appropriate security measures in place. Hard copy records will be stored in a locked cabinet in a secure location. Access to records and data will be limited to study team members only. Study data will be de-identified and a master linking log with identifiers will be kept and stored separately from the data.

Data availability statement

The data from this study will be available from the corresponding author upon reasonable request.

Ethics and dissemination

This study abided by the Declaration of Helsinki and was approved by the SingHealth Central Institutional Review Board (2023/2170). Written informed consent will be obtained from all study participants.

We aim to publish the results of the study in peer-reviewed scientific journals and present them at national and international scientific conferences. If desired, participants will be informed about the outcomes of the study.

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Contributors QYH—conceptualisation. QYH, CMDL, ITL, LKL, SJC, ST, S-YCT, LLEO and TK—methodology and data collection. QYH—data analysis and interpretation. QYH, CMDL, ITL, SJC, LLEO, CST and TK—funding acquisition. CST and TK—supervision. QYH will write the first draft and all authors will participate in revising the manuscript.

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Competing interests None declared.

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