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Prospective Study of Long Noncoding RNA, MGAT3-AS1, and Viremia of BK Polyomavirus and Cytomegalovirus in Living Donor Renal Transplant Recipients

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Introduction: Viremia after renal transplantation is a major cause of morbidity and mortality and treatment opportunities are limited. Tests to determine the increased risk for viremia would be preferable.

Methods: In a prospective, single-center study, we conducted follow-up of 163 renal transplant recipients after incident living donor renal transplantation. We determined a long noncoding RNA, β-1,4-mannosylglycoprotein 4-β-N-acetylglucosaminyltransferase-antisense1 (MGAT3-AS1/beta-actin ratio), in peripheral blood mononuclear cells. Viremia of BK polyomavirus and cytomegalovirus was diagnosed with more than 1000 plasma copies/ml within the first 3 postoperative months. The MGAT3-AS1/beta-actin ratio was assessed before viremia was determined.

Results: Receiver operator characteristics curve analysis showed a median MGAT3-AS1/beta-actin ratio cutoff value of 4.45×10^{-6} to indicate viremia after transplantation. Samples for 11 of 66 renal transplant recipients (17%) with MGAT3-AS1/beta-actin ratios below 4.45×10^{-6} showed viremia of BK polyomavirus and cytomegalovirus compared with only 6 of 97 renal transplant recipients (6%) with higher MGAT3-AS1/beta-actin ratios (odds ratio [OR]: 3.03; 95% confidence interval [CI]: 1.06–8.67 by Fisher exact test). Furthermore, samples for 6 of 66 renal transplant recipients (9%) with MGAT3-AS1/beta-actin ratios below 4.45×10^{-6} showed BK polyomavirus viremia compared with none of 97 renal transplant recipients (0%) with higher MGAT3-AS1/beta-actin ratios (OR: 20.95; 95% CI, 1.16–378.85 by Fisher exact test). Multivariate logistic regression analysis confirmed that MGAT3-AS1/beta-actin ratios below the cutoff level remained significantly associated with viremia after transplant. Lower MGAT3-AS1/beta-actin ratios occurred with rituximab-containing induction therapy.

Conclusions: A low MGAT3-AS1/beta-actin ratio indicates an increased risk for viremia of BK polyomavirus and cytomegalovirus in living donor renal transplant recipients.

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Infections are a common cause of morbidity and mortality after solid organ transplantation.[1] In particular, activation of latent, opportunistic infections including BK polyomavirus and cytomegalovirus occurs within the first months after renal transplantation.[1–3] Reactivation of virus replications is expected to reflect the effects of immunosuppressive therapies.[3,4] Cytomegalovirus replication can be observed in 10% of transplant recipients even in the presence of prophylaxis with valganciclovir.[3,5,6] Importantly, cytomegalovirus infection within 100 days of transplant is an independent risk factor for overall and cardiovascular mortality.[7] Replication of BK polyomavirus, which is detected in 10% to 30% of renal transplant recipients within the first months after transplantation, is an important cause of premature allograft failure.[8,9] In the absence of established antiviral drugs, follow-up in renal transplant recipients mainly includes careful screening for BK polyomavirus and preemptive reduction of immunosuppression. However, simple noninvasive tests to determine increased risk for viremia associated with the immunosuppressive regimes are not established yet.

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Long noncoding RNAs have been shown to play an important role on the innate and adaptive immune system.[10] The expression of several long noncoding RNAs defines virus-specific effector and memory cells.[11] A long noncoding RNA, β-1,4-mannosylglycoprotein 4-β-N-acetylglucosaminyl-transferaseantisense-1 (MGAT3-AS1), has been described as a key molecule for the innate immune system.[12] Peripheral blood mononuclear cells, including adaptive and acquired immune responses of B and T lymphocytes, are important for controlling virus replication both in BK polyomavirus and cytomegalovirus.[13–15] As several immunosuppressive regimes largely affect peripheral blood mononuclear cells, we reasoned that MGAT3-AS1 may characterize immunosuppressive therapies.

We investigated the hypothesis that determination of the MGAT3-AS1/beta-actin ratio in peripheral blood mononuclear cells during the first postoperative month predicts viremia of BK polyomavirus and cytomegalovirus within the first 3 postoperative months in living donor renal transplant recipients. We conducted a prospective single-center study in renal transplant recipients who were treated with tacrolimus, mycophenolate, and induction therapy according to patients' immunologic risks for rejection, including combinations of basiliximab, rituximab, prednisolone, and thymoglobulin.

METHODS

Study Design and Participants

The ongoing Molecular Biological and Moleculargenetic Monitoring of Therapy After Kidney Transplantation (MoMoTxRes) study continuously recruits incident renal transplant recipients at Odense University Hospital, Denmark (ClinicalTrials.gov number NCT01515605). Details from the study design had been published previously.[16,17] The study protocol was in accordance with the ethical standards of the Declarations of Helsinki and Istanbul. The study was approved by the local ethics committee (Den Videnskabsetiské Komite for Region Syddanmark, Projekt-ID 20100098). Written informed consent was obtained from all patients before entry into the study. Inclusion criteria were renal transplant recipients with incident living donor renal transplantation and participation in the MoMoTxRes study. Exclusion criteria were age younger than 18 years or missing informed consent. Baseline characteristics of donors and recipients and information on organ procurement were obtained from medical records. Between January 2011 and June 2017, complementary DNA was obtained from peripheral blood mononuclear cells in 163 renal transplant recipients. The induction therapy consisted of basiliximab in 91 recipients (56%); basiliximab and prednisolone in 2 recipients (1%); basiliximab, rituximab, and prednisolone in 21 recipients (13%); rituximab and prednisolone in 19 recipients (12%); rituximab, prednisolone, and thymoglobulin in 23 recipients (14%); thymoglobulin in 2 recipients (1%) and prednisolone and thymoglobulin in 5 recipients (3%), respectively.

Virologic Studies

DNA was extracted from ethylenediamine tetraacetic acid–anticoagulated plasma samples using DNA and viral NA large volume kit (Roche Diagnostics, Hvidovre, Denmark). Viral load was determined by quantitative polymerase chain reactions (PCRs) using a LightCycler 480 instrument (Roche Diagnostics).[18,19] For quantification of cytomegalovirus the UL55 assay (Promega) was used.[18] The performance of each assay was verified twice a year with a virus-specific proficiency panel (QCMD, Glasgow, Scotland). For BK polyomavirus the *VP2* gene was targeted; primers and probe were designed by Primer Express 3.0 (Thermo Scientific, Waltham, MA) and consisted of: forward, ACACCTGCTCTTGAAGCATATGAA; reverse CTTTTATTAGTAGTTTTGGCACTTGCA, and probe FAM-CTCCCAAAAAGCC-MGB. We performed 15-μl reactions containing LightCycler 480 Probes Master (Roche Applied Science), 1000 nM of each primer, 200 nM of the probe, and 5 μL DNA eluate. The amplification protocol was as follows: 10 minutes at 95 °C, followed by 45 cycles of 95 °C for 10 seconds, 60° C for 30 seconds, and 72° C for 1 second. Assay validation was performed using commercial standards (HCMV, AD169 Strain, Quantitated Viral DNA; BK Virus, MM Strain, Quantitated Viral DNA; Advanced Biotechnologies Inc., Eldersburg, MD USA). Obtained Cq values were quantified using external standard curves. The y-intercept value was calculated by a calibrator that was pipetted together with the actual PCR targets. Acceptable deviation for the calibrator was set to 1 quantification cycle (Cq) value.

Measurements of Long Noncoding RNA, MGAT3-AS1

We used modifications of our previously described measurements.[16] Briefly, mononuclear cells were repeatedly obtained from heparinized peripheral blood at days 1, 8, 15, 22, and 29 post-transplant. Peripheral blood mononuclear cells were obtained by density centrifugation using Histopaque (Sigma-Aldrich, St. Louis, MO; density 1.077 g/ml); and the cell interphase was washed by centrifugation in Hanks balanced salt solution and suspended in TRIzol (TRI Reagent, Sigma Aldrich). Total RNA was isolated using the

RNeasy Mini kit including RNase-free DNase set (Qiagen, Hilden, Germany).[16] Complementary DNA was synthesized from 300 ng of total RNA by using a QuantiTect Reverse Transcription kit (Qiagen). Genomic DNA was eliminated by incubation of each RNA sample with the genomic DNA elimination mix for 4 minutes at 42 °C followed by incubation with the manufacturer mix of QuantiTect Reverse Transcription primer, reverse transcriptase, and reverse transcription buffer for 60 minutes at 37 °C, followed by heating to 95 °C for 5 minutes.[16]

Transcripts of MGAT3-AS1 and β-actin were measured with quantitative reverse transcription PCRs using the following primers[16,20]: MGAT3-AS1, 5′GTAGACCATGACACCCAGCA3′; 5′CTCGTCTCTGCTCCATGGTGA3′; and β-actin, 5′GGACTTCGAGCAAGAGATGG3′, 5′AGCACTGTGTTGGCGTACAG3′.

Quantitative reverse transcription PCR was performed using 5 μL of single-stranded complementary DNA that was added to a final volume of 20 μL, which contained 10 μL FastStart Essential DNA Green Master mix (Roche Diagnostics), and 500 nmol/L of each primer.[16] Preincubation at 95 °C for 10 minutes was followed by 55 cycles with denaturation at 95 °C for 10 seconds, annealing at 63 °C for 10 seconds, and extension at 72 °C for 10 seconds. The sizes of PCR products for MGAT3-AS1 and beta-actin transcripts were 158 bp and 234 bp, respectively.[16] Cq values for each reaction were determined using LightCycler 96 Software 1.1 (Roche Diagnostics).[16] The target gene expressions were determined relative to the housekeeping gene beta-actin, and normalized ratios of transcript expression were calculated according to the following equation: Normalized ratio = $ET^{(CqR-CqT)}$, where ET is the efficiency of target amplification and CqT and CqR are Cqs at target and reference detection, respectively.[16] We also used the delta Cq method with MGAT3-AS1 and beta-actin transcripts from recipients and controls for analysis. The treating physicians were unaware of the results of the MGAT3-AS1/beta-actin ratio results.

Outcome

The primary outcome was detection of more than 1000 copies/ml of BK polyomavirus DNA or cytomegalovirus DNA in plasma from a living donor renal transplant recipient. The cutoff value of 1000 copies/ml was recently described for our assay (Hoegh S, Skov M, Kemp M. Development of an in-house real-time PCR for monitoring BK viral load in renal allograft recipients [abstract] 2016; ECCMID 2016 ePoster EV0108). Testing for BK polyomavirus DNA and cytomegalovirus DNA was performed according to institutional protocols for follow-up of living donor renal transplant recipients. Plasma samples were obtained during routine monthly outpatient visits for the first 3 months after transplantation, as well as whenever patients were hospitalized, allograft function declined, and the treating physician suspected viremia. If more than 1000 copies/ml were detected on several occasions in the same recipient, only the first detection was used for the analysis of the primary outcome.

Statistical Analysis

Continuous data are presented as median and interquartile range (IQR) or range, as indicated. Frequency counts were calculated for categorical data. Contingency tables were analyzed using the Fisher exact test or χ^2 test as appropriate. For continuous variables, nonparametric Kruskal-Wallis tests or nonparametric Mann-Whitney tests were performed as appropriate. We performed receiver operating characteristic curve analysis to detect the accuracy of the MGAT3-AS1/beta-actin ratio to predict viremia. The cutoff level was determined using the Youden index.

Logistic regression analysis was performed using a forward stepwise method to analyze the effect of variables on the outcome of viremia of BK polyomavirus and cytomegalovirus. Categorical variables were recipient sex, cardiovascular disease, diabetes mellitus, hypertension, immunosuppression regime, delayed graft function, and donor sex.

Data were analyzed using GraphPad Prism software (version 6.0, GraphPad Software, La Jolla, CA) and IBM SPSS Statistics (version 26; Armonk, NY). All statistical tests were 2-sided. Two-sided P values less than 0.05 were considered indicate statistically significant.

RESULTS

Baseline Characteristics

We prospectively followed up 163 incident living donor renal transplant recipients who were treated with tacrolimus and mycophenolate for 3 months after transplant. The characteristics of recipients are shown in Table 1. Of the recipients, 110 (67%) were male and 53 (33%) were female; 22 (13%) had had previous transplants. All patients were treated with the calcineurin inhibitor, tacrolimus (0.5 mg/kg daily). Target trough levels for plasma tacrolimus were 8 to 15 ng/ml during the first 3 postoperative months. All patients were treated with antiproliferative mycophenolate (i.e., mycophenolate mofetil, 1000 mg twice daily; or mycophenolate sodium, 720 mg twice daily). Induction therapies were used according to patients' immunologic risks for rejection according to our institutional protocols. Induction therapies included combinations of anti–interleukin 2 receptor antibody (basiliximab, 20 mg at day 0 pretransplant and day 4 post-transplant; $n = 114$, 70%), anti–B-cell CD20 antibody (rituximab,

Table 1. Clinical characteristics of 163 recipients of renal allograft stratified according to post-transplant viremia

Characteristic	All patients ($N = 163$)	Post-transplant viremia ($n = 17$)	No post-treatment viremia ($n = 146$)	*P* value (viremia vs. no viremia)
Age of recipient, yr	45 (36–56)	43 (40–61)	45 (36–66)	0.44
Recipient sex male, *n* (%)	110 (66%)	10 (59%)	100 (68%)	0.42
Body weight, kg	81 (72–95)	77 (66–82)	83 (73–96)	0.09
Body mass index, kg/m^2	27 (24–30)	27 (24–28)	27 (24–31)	0.39
Systolic blood pressure, mm Hg	148 (130–161)	146 (122–165)	149 (130–163)	0.57
Diastolic blood pressure, mm Hg	86 (76–96)	82 (74–94)	87 (76–96)	0.43
Cause kidney disease, *n* (%)				0.91
Glomerulonephritis	71 (44%)	7 (41%)	64 (44%)	
Diabetes mellitus	22 (13%)	1 (6%)	21 (14%)	
Hypertension	18 (11%)	2 (12%)	16 (11%)	
Interstitial nephritis	14 (9%)	2 (12%)	12 (8%)	
Polycystic kidney disease	18 (11%)	2 (12%)	16 (11%)	
Other/unknown	20 (12%)	3 (17%)	17 (12%)	
Duration of dialysis, mo	10 (1-27)	10 (2-24)	10 (1-19)	0.58
Type of dialysis, *n* (%)				0.87
Hemodialysis	85 (52%)	8 (47%)	77 (53%)	
Peritoneal dialysis	40 (25%)	5 (29%)	35 (24%)	
Preemptive transplant	38 (23%)	4 (24%)	34 (23%)	
Age of donor, yr	52 (46–58)	58 (48–62)	51 (44–58)	0.12
Donor sex male, *n* (%)	68 (42%)	8 (47%)	60 (41%)	0.80
No. of HLA mismatches (range, 0–8)	3 (2–5)	3 (0–6)	3 (2–5)	0.76
Transplantation, *n* (%)				0.71
First transplant	141 (87%)	14 (82%)	127 (87%)	
Second or more transplant	22 (13%)	3 (18%)	19 (13%)	
ABO blood type incompatibility, *n* (%)	48 (29%)	5 (29%)	43 (29%)	1.00
Cytomegalovirus serostatus, *n* (%)[a]				0.58
Specific antibodies positive	108 (66%)	12 (75%)	96 (66%)	
Specific antibodies negative	54 (33%)	4 (25%)	50 (33%)	
Induction therapy, *n* (%)				0.03
Basiliximab	114 (70%)	7 (41%)	107 (73%)	
Rituximab	63 (39%)	11 (65%)	52 (36%)	
Prednisolone	70 (43%)	11 (65%)	59 (40%)	
Thymoglobulin	30 (18%)	7 (41%)	23 (16%)	
Plasma creatinine pretransplant, μmol/l[b]	751 (615–997)	651 (560–1027)	754 (619–1002)	0.60

HLA, human leukocyte antigen.
Continuous data are presented as median (interquartile range). Categorical data are presented as number (percent). Groups containing continuous data were compared using Mann-Whitney test, whereas groups containing categorical data were compared using Fisher exact test or χ^2 test, as appropriate.
[a]Cytomegalovirus serostatus was not available for 1 recipient before transplant.
[b]To convert the values for creatinine to milligram per deciliter, divide by 88.4.

375 mg/m^2, 4 weeks pretransplant; $n = 63$; 39%), prednisolone (25 mg daily; $n = 70$; 43%), and T-cell–depleting thymoglobulin (1.5 mg/kg daily for 4 days; $n = 30$; 18%). All living donor renal transplant recipients received prophylaxis for 3 months with either 200 mg acyclovir daily or 450 mg valganciclovir daily. Seventy-eight transplant recipients (48%) received β-blockers, 58 recipients (38%) received calcium channel agonists, and 62 recipients (38%) received diuretics. Eleven recipients (7%) had delayed graft function (i.e., at least 1 dialysis session in the first postoperative week).

Postoperative Viremia of BK Polyomavirus and Cytomegalovirus

During the first 3 postoperative months viremia of BK polyomavirus, cytomegalovirus, or both was observed in 17 of 163 living donor renal transplant recipients (10%). Because 2 recipients had viremia of both BK polyomavirus virus and cytomegalovirus within the first 3 months after transplant, we observed 19 episodes of viremia in 17 patients; 6 recipients (4%) had BK polyomavirus virus, whereas 13 recipients (8%) had cytomegalovirus, and only the first detection was used for the analysis of the primary outcome. As shown in Table 1, recipients in whom viremia developed within the first 3 postoperative months had higher percentages of induction therapy containing rituximab, and prednisolone or thymoglobulin or both. Table 2 shows the univariate analysis of clinical factors for developing viremia providing the odds ratios. We did not identify an association of viremia with other variables, including human leukocyte antigen mismatches, delayed graft function, lymphocyte count, or allograft function 1 month after transplant. The number of

Table 2. Univariate logistic regression analysis of factors for developing viremia post-transplant.

Characteristic	Univariate analysis
Age, yr	1.01 (0.94–1.07)
Sex (1 = male)	1.36 (0.25–7.29)
Diabetes (1 = yes)	0.30 (0.19–4.57)
Hypertension (1 = yes)	1.28 (0.17–9.85)
Cardiovascular disease (1 = yes)	0.70 (0.08–6.48)
Basiliximab and prednisone[a]	0.00[b]
Basiliximab, rituximab, and prednisone	3.72 (0.33–42.27)
Rituximab and prednisone	5.34 (0.64-44.89)
Rituximab, prednisone, and thymoglobulin	15.99 (1.49–171.63)
Thymoglobulin	26.77 (0.44-1645.85)
Prednisone and thyroglobulin	0.00[b]
Systolic blood pressure, mm Hg	0.61 (0.26–1.41)
Diastolic blood pressure, mm Hg	0.38 (0.07-2.09)
Previous transplant	0.87 (0.30–2.59)
Donor sex (1 = male)	2.73 (0.61-12.17)
Delayed graft function (1 = yes)	0.31 (0.00–21.4)
HLA_AB mismatch (number)	0.97 (0.44–2.17)
HLA_QR mismatch (number)	0.64 (0.15–2.70)
Estimated glomerular filtration rate 1 month post-transplant	0.98 (0.93–1.02)

HLA, human leukocyte antigen, major histocompatibility complex.
Data are expressed as the odds ratio (95% confidence interval).
[a]Basiliximab alone denotes reference.
[b]95% Confidence Interval not available.

human leukocyte antigen mismatches between donor and recipient did not differ in 17 recipients with viremia compared with 146 recipients without viremia ($P = .76$). The sample for 1 of 11 recipients (9%) with delayed graft function showed viremia, whereas samples for 16 of 152 recipients (11%) with immediate graft function showed viremia (OR: 0.86; 95% CI: 0.10–7.1 by Fisher exact test). Lymphocyte counts at the first postoperative day did not differ in recipients with and without viremia (median [range], 0.86/nl [0.05–1.82] vs. 1.01/nl [0.05–3.36]; $P = 0.47$). There was no association of lymphocyte counts and MGAT3-AS1/beta-actin ratio at the first postoperative day ($r = 0.18$ by nonparametric Spearman correlation). At 1 month after transplant, allograft functions did not differ in recipients with and without viremia (estimated glomerular filtration rate, median [range], 48.5 ml/min/1.73cm^2 [12.9–79.2] vs. 50.1 ml/min/1.73cm^2 [4.9 to 97.7]; $P = 0.63$). Median plasma tacrolimus levels were similar in patients in whom viremia developed viremia (12.5 ng/ml; IQR, 8.8–17.5) and those without viremia (15.1 ng/ml, IQR 12.5–17.7; $P = 0.82$ by Mann-Whitney test). Of the 163 recipients, 108 (66%) had cytomegalovirus-specific antibodies measured in samples obtained before transplantation. Thirteen recipients had viremia of cytomegalovirus after transplantation, 10 recipients with cytomegalovirus viremia were seropositive, 2 were seronegative, and the status of 1 was unknown before transplantation. Among the 2 seronegative recipients with viremia of cytomegalovirus after transplantation, 1 had a seronegative donor and 1 had a seropositive donor. Data for BK polyomavirus-specific antibodies were not available before transplantation. Two of 17 renal transplant recipients with viremia (12%) exhibited rejection compared with 17 of 146 recipients without viremia (12%) (OR: 1.01; 95% CI: 0.21–4.82 by Fisher exact test).

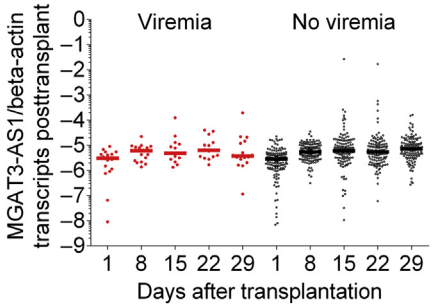

Figure 1. Scatter diagram showing long noncoding RNA, β-1,4-mannosylglycoprotein 4-β-N-acetylglucosaminyl-transferaseantisense-1 (MGAT3-AS1)/beta-actin ratio in peripheral blood mononuclear cells from 163 living donor renal transplant recipients with and without post-transplant viremia during the first postoperative month. Median levels are indicated by vertical lines.

Long noncoding RNA, MGAT3-AS1, and viremia

The median (IQR) MGAT3-AS1/beta-actin ratio in 163 renal transplant recipients during the first post-operative month was 4.83×10^{-6} (3.07×10^{-6} to 8.40×10^{-6}). As a group, the median MGAT3-AS1/beta-actin ratio in recipients with viremia (4.01×10^{-6}; IQR, 2.66×10^{-6} to 8.55×10^{-6}), without viremia (5.11×10^{-6}; IQR, 3.15×10^{-6} to $6.85^{*}10^{-6}$), healthy subjects (4.85×10^{-6}; IQR, 3.08×10^{-6} to 6.85×10^{-6}), and patients with end-stage renal disease (4.55×10^{-6}; IQR, 2.25×10^{-6} to $8.52^{*}10^{-6}$) did not differ ($P = 0.43$ by Kruskal-Wallis-test). Figure 1 shows the MGAT3-AS1/beta-actin ratio measured at different time points after transplant in recipients with and without post-transplant viremia.

Receiver operator characteristics curve analysis showed a median MGAT3-AS1/beta-actin ratio cutoff value of 4.45 0215 10^{-6} to indicate viremia. Samples for 11 of 66 renal transplant recipients (17%) with MGAT3-AS1/beta-actin ratios below 4.45×0^{-6} within the first postoperative month showed viremia of BK polyomavirus and cytomegalovirus compared with only 6 of 97 renal transplant recipients (6%) with higher MGAT3-AS1/beta-actin ratio (OR: 3.03; 95% CI: 1.06–8.67 by Fisher exact test). Samples for 7 of 66 renal transplant recipients (11%) with MGAT3-AS1/beta-actin ratios below 4.45×10^{-6} showed viremia of cytomegalovirus compared with 6 of 97 renal transplant recipients (6%) with higher MGAT3-AS1/beta-actin ratios (OR: 1.80; 95% CI: 0.58–5.61 by Fisher exact test).

Table 3. Clinical characteristics of 163 recipients of renal allograft stratified according to long noncoding RNA, MGATS-AS1

Characteristic	Recipients with MGAT3-AS1/beta-actin ratio below cutoff (n = 66)	Recipients with MGAT3-AS1/beta-actin ratio above cutoff (n = 97)	P value
Age of recipient, yr	49 (40–61)	43 (33–62)	0.01
Recipient sex male, n (%)	41 (62%)	69 (71%)	0.24
Body weight, kg	81 (72–93)	82 (73–99)	0.24
Body mass index, kg/m^2	27 (23–29)	27 (24–32)	0.42
Systolic blood pressure, mm Hg	144 (129–161)	150 (130–165)	0.39
Diastolic blood pressure, mm Hg	82 (74–95)	89 (79–96)	0.06
Cause of kidney disease, n (%)			0.18
Glomerulonephritis	27 (41%)	44 (45%)	
Diabetes mellitus	8 (12%)	14 (15%)	
Hypertension	8 (12%)	10 (10%)	
Interstitial nephritis	6 (9%)	8 (8%)	
Polycystic kidney disease	12 (18%)	6 (6%)	
Other/unknown	5 (8%)	15 (16%)	
Duration of dialysis, mo	12 (5–28)	8 (0–16)	0.05
Type of dialysis, n (%)			0.07
Hemodialysis	41 (62%)	44 (45%)	
Peritoneal dialysis	15 (23%)	25 (26%)	
Preemptive transplant	10 (15%)	28 (29%)	
Age of donor, yr	51 (44–58)	52 (45–59)	0.99
Donor sex male, n (%)	21 (32%)	47 (48%)	0.04
Number of HLA mismatches, range, 0–8	4 (2–5)	3 (2–5)	0.39
Transplantation, n (%)			0.06
First transplant	53 (80%)	88 (91%)	
Second or more transplants	13 (20%)	9 (9%)	
ABO blood type incompatibility, n (%)	28 (42%)	20 (21%)	0.01
Cytomegalovirus serostatus, n (%)[a]			0.40
Specific antibodies positive	46 (70%)	62 (64%)	
Specific antibodies negative	19 (29%)	35 (36%)	
Induction therapy, n (%)			0.001
Basiliximab	36 (55%)	78 (80%)	
Rituximab	43 (65%)	20 (21%)	
Prednisolone	43 (65%)	27 (28%)	
Thymoglobulin	20 (30%)	10 (10%)	
Plasma creatinine pretransplant, µmol/l[b]	789 (619–986)	732 (605–1011)	0.75
Post-transplant viremia in all immunosuppressive regimes, n (%)			0.04
Viremia	11 (17%)	6 (6%)	
No viremia	55 (83%)	91 (94%)	
Post-transplant viremia in rituximab-containing regime, n (%)			0.02
Viremia	10 (23%)	0 (0%)	
No viremia	33 (77%)	20 (100%)	

The MGAT3-AS1/beta-actin ratio used the cutoff level of 4.45×10^{-6}. Continuous data are presented as median (interquartile range). Categorical data are presented as number (percent). Groups containing continuous data were compared using Mann-Whitney test, whereas groups containing categorical data were compared using Fisher exact test or χ^2 test, as appropriate.
[a]Cytomegalovirus serostatus was no available for 1 recipient before transplant.
[b]To convert the values for creatinine to milligram per deciliter, divide by 88.4.

Samples for 6 of 66 renal transplant recipients (9%) with MGAT3-AS1/beta-actin ratios below 4.45×10^{-6} showed BK polyomavirus viremia compared with none of 97 renal transplant recipients (0%) with higher MGAT3-AS1/beta-actin ratios (OR: 20.95; 95% CI, 1.16–378.85 by Fisher exact test).

Samples for 8 of 54 renal transplant recipients (15%) in the lowest MGAT3-AS1/beta-actin ratio tertile, 4 of 54 (7%) in the middle tertile, and 5 of 55 (9%) in the highest tertile showed viremia (χ^2 7.45; $P = 0.41$ by χ^2 test). Samples for 7 of 41 renal transplant recipients (17%) in the lowest MGAT3-AS1/beta-actin ratio quartile, 5 of 40 (13%) in the second quartile, none of 41 (0%) in the third quartile, and 5 of 41 (12%) in the highest quartile showed viremia ($\chi^2 = 7.03$; $P = 0.07$ by χ^2 test). These analyses confirmed that the MGAT3-AS1/beta-actin ratio cutoff of 4.45×10^{-6}, which was determined by receiver operating characteristic curve and using the Youden index, provided the best discrimination to detect viremia.

A lower MGAT3-AS1/beta-actin ratio determined in 142 recipients 1 month after transplantation showed an increased risk for viremia. Samples for 10 of 47 renal transplant recipients (21%) with MGAT3-AS1/beta-actin ratios below 4.45×10^{-6} determined 1 month after transplantation showed viremia of BK polyomavirus and cytomegalovirus compared with only 6 of 95 renal transplant recipients (6%) with higher MGAT3-AS1/beta-actin ratio (OR: 4.00; 95% CI: 1.36–11.84 by Fisher exact test).

In addition, we obtained similar results using the delta Cq method, showing that recipients with lower MGAT3-AS1 levels are at increased risk for viremia. Samples for 7 of 35 renal transplant recipients (20%) with MGAT3-AS1 levels below the cutoff value of 0.584 showed viremia of BK polyomavirus and cytomegalovirus compared with only 10 of 128 renal transplant recipients (8%) with higher MGAT3-AS1 levels (OR: 2.95; 95% CI: 1.03–8.43 by Fisher exact test).

As shown in Table 3, recipients with MGAT3/beta-actin ratios below the cutoff value of 4.45×10^{-6} were older ($P = 0.01$), had a higher percentage of ABO blood type–incompatible renal transplant ($P = 0.01$), and had

Table 4. Multivariate logistic regression analysis of factors for developing post-transplant viremia

	Logistic regression step 0		Logistic regression step 1	
Variables	Score	Significance	Score	Significance
Constant		0.00		0.00
MGAT3-AS1/beta-actin ratio below cutoff	4.62	0.03		0.04
Age, yr	0.77	0.38	0.19	0.66
Sex (1 = male)	0.65	0.42	0.37	0.55
Diabetes (1 = yes)	1.44	0.23	1.69	0.19
Hypertension (1 = yes)	0.20	0.66	0.23	0.63
Cardiovascular disease (1 = yes)	1.15	0.77	1.20	0.75
Basiliximab	13.18	0.04	8.52	0.20
Basiliximab and prednisone	0.24	0.63	0.14	0.71
Basiliximab, rituximab, and prednisone	0.02	0.88	0.31	0.58
Rituximab and prednisone	0.66	0.42	0.37	0.55
Rituximab, prednisone, and thymoglobulin	7.03	0.01	3.18	0.07
Thymoglobulin	3.39	0.07	3.07	0.08
Prednisone and thymoglobulin	0.60	0.44	0.35	0.56
Systolic blood pressure, mm Hg	0.76	0.38	0.62	0.43
Diastolic blood pressure, mm Hg	0.41	0.52	0.12	0.73
Previous transplant	1.83	0.18	0.85	0.36
Donor sex (1 = male)	0.65	0.42	1.76	0.18
Delayed graft function (1 = yes)	0.01	0.95	0.01	0.93
HLA_AB mismatch (number)	0.00	1.00	0.01	0.91
HLA_QR mismatch (number)	0.36	0.55	0.48	0.49
Estimated glomerular filtration rate 1 month after transplant	0.42	0.52	0.45	0.50

HLA, human leukocyte antigen; MGAT3-AS1, β-1,4-mannosylglycoprotein 4- β-N-acetylglucosaminyltransferase-antisense 1.

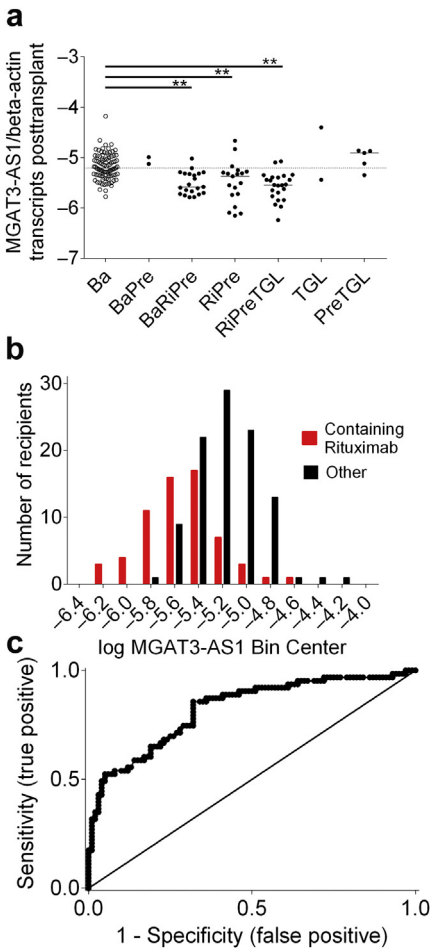

Figure 2. Long noncoding RNA, β-1,4-mannosylglycoprotein 4-β-N-acetylglucosaminyl-transferaseantisense-1 (MGAT3-AS1)/beta-actin ratio in peripheral blood mononuclear cells from 163 living-donor renal transplant recipients during the first postoperative month. (a) Scatter diagram showing MGAT3-AS1/beta-actin ratio in recipients with induction therapy consisting of basiliximab (Ba; median, 6.21×10^{-6}; IQR, 4.47×10^{-6} to 10.07×10^{-6}; $n = 91$); basiliximab and prednisolone (BaPre; median, 8.84×10^{-6}; IQR, 7.50×10^{-6} to 10.18×10^{-6}; $n = 2$); basiliximab, rituximab, and prednisolone (BaRiPre; median, 2.62×10^{-6}; IQR, 1.94×10^{-6} to 4.82×10^{-6}; $n = 21$); rituximab and prednisolone (RiPre; median, 4.25×10^{-6}; IQR, 1.81×10^{-6} to 5.28×10^{-6}; $n = 19$); rituximab, prednisolone, and thymoglobulin (RiPreTGL; median, 2.87×10^{-6}; IQR, $1.71*10^{-6}$ to 4.06×10^{-6}; $n = 23$); thymoglobuline (TGL; median, 21.67×10^{-6}; $n = 2$); prednisolone and thymoglobulin (PreTGL; median, 12.31×10^{-6}, IQR, 8.06×10^{-6} to 113.63×10^{-6}; $n = 5$). Median levels were indicated by vertical lines. The dotted line indicates the median level for the induction therapy with basiliximab. ** indicates $P < 0.01$ between the groups as assessed by Kruskal-Wallis test and Dunn multiple comparisons test. (b) Histogram of MGAT3-AS1/beta-actin ratio in recipients with induction therapy containing rituximab ($n = 63$ and other induction therapy ($n = 100$). Most recipients with induction therapy containing rituximab had levels below –4.45 (red bars) whereas recipients with other induction therapy showed levels ranging from –5.8 to –4.2. (c) Receiver operator characteristics curve showing that the MGAT3-AS1/beta-actin ratio characterizes the immunosuppressive regime containing rituximab. The curve indicates that MGAT3-AS1/beta-actin ratio characterizes the immunosuppressive regime containing rituximab. Area under curve, 0.82 (95 percent confidence interval: 0.76–0.89; $P < 0.001$). IQR, Interquartile range; MGAT3-AS1 Long noncoding RNA, β-1,4-mannosylglycoprotein 4-β-N-acetylglucosaminyl-transferase antisense-1.

higher percentages of induction therapy containing rituximab, and prednisolone, thymoglobulin, or both ($P = 0.001$). At diagnosis onset the median cytomegalovirus titer was 3300 in the group with MGAT3/beta-actin ratios below the cutoff value and 3667 with MGAT3/beta-actin ratios above the cutoff value ($P = 0.72$ by Mann-Whitney test). All patients with polyoma BK virus had MGAT3/beta-actin ratios below the cutoff point. At diagnosis onset median polyoma BK virus titer was 163,500.

Table 4 shows variables in the multivariable logistic regression analysis. Only MGAT3-AS1/beta-actin ratios below 4.45×10^{-6} remained significantly associated with post-transplant viremia.

Figure 2 shows that the MGAT3-AS1/beta-actin ratio was significantly lower in renal transplant recipients who received immunosuppressive regimes containing rituximab (each $P < 0.01$ by Kruskal-Wallis test and Dunn multiple comparisons test). Table 5 shows the effect of the induction therapy on viremia. MGAT3-AS1/beta-actin ratios below the cutoff value predicted viremia both in the entire group and in recipients receiving rituximab. Among the recipients who

Table 5. Induction therapy in recipients with living donor renal transplant, MGAT3-AS1/beta-actin ratio, and viremia

	All recipients			Recipients with MGAT3-AS1/beta-actin ratio[a] below cutoff		
Induction therapy, *n* (%)	Viremia ($n = 17$)	No viremia ($n = 146$)	P value[b]	Viremia ($n = 11$)	No viremia ($n = 55$)	*P* value[b]
Basiliximab	5 (29)	86 (59)	Reference	1 (9)	21 (38)	Reference
Basiliximab and prednisone	0 (0)	2(1)	1.00	0 (0)	0 (0)	1.00
Basiliximab, rituximab, and prednisone	2 (12)	19 (13)	0.61	2 (18)	12 (22)	0.55
Rituximab and prednisone	3 (18)	16 (11)	0.14	2 (18)	8 (14)	0.22
Rituximab, prednisone, and thymoglobulin	6 (35)	17 (12)	0.01	6 (55)	13 (24)	0.03
Tthymoglobulin	1 (6)	1 (1)	0.13	0 (0)	1 (2)	1.00
Prednisone and thymoglobulin	0 (0)	5 (3)	1.00	0 (0)	0 (0)	1.00

MGAT3-S1, Long nonoding RNA, β-annosylglycoprotein 4-beta;-cetylglucosaminyl-ransfera
[a]The MGAT3-AS1/beta-actin ratio cutoff level was 4.45×10^{-6}.
[b]*P* values were calculated using Fisher exact test where induction therapy with basiliximab was chosen as reference.

received rituximab, samples for 10 of 43 renal transplant recipients (23%) with MGAT3-AS1/beta-actin ratios below 4.45×0^{-6} showed viremia compared with none of 20 renal transplant recipients (0%) with higher MGAT3-AS1/beta-actin ratios ($P = 0.02$ by Fisher exact test). Compared with induction therapy with basiliximab, we observed an increased risk for viremia in recipients with induction therapy containing rituximab, prednisolone, and thymoglobulin ($P = 0.01$). Importantly, viremia was shown in all 6 of 6 recipients (100%) with induction therapy containing rituximab, prednisolone, and thymoglobulin, and all had MGAT3-AS1/beta-actin ratios below the cutoff level of 4.45×10^{-6} (negative predictive value, 1.00; 95% CI: 0.40–1.00).

DISCUSSION

We showed that living donor renal transplant recipients with long noncoding RNA, MGAT3-AS1/beta-actin ratios below the cutoff level have an increased risk for post-transplant viremia. The ORs for recipients with median MGAT3-AS1/beta-actin ratios during the first postoperative month below the cutoff level was 3.03 for post-transplant viremia of BK polyomavirus and cytomegalovirus. Multivariate logistic regression analysis confirmed that a MGAT3-AS1/beta-actin ratio below the cutoff level remained significantly associated with viremia.

There is biological evidence that long noncoding RNA MGAT3-AS1 in peripheral blood mononuclear cells may be the marker of immunosuppression after transplantation. MGAT3-AS1 is the regulator of glycoprotein oligosaccharides in mononuclear cells, which are involved in innate immunity. Recently *Shen et al.*[21] explored possible mechanisms of long-noncoding RNA MGAT3-AS1 (also called "TapSaki") in lipopolysaccharide-induced human kidney 2 cells.[21] They showed that MGAT3-AS1 promoted the expression of the phosphatase and tensin homolog (*PTEN*) gene and that it activated the toll-like receptor 4/nuclear factor kappa B pathway–related proteins involved in immune responses. Using in vivo experiments they showed that MGAT3-AS1 knockdown regulated phosphatase and tensin homolog and decreased renal function.[21] Future studies may indicate that MAG3-AS1 can regulate these pathways also in humans. Currently it is unknown whether the *PTEN* gene and toll-like receptor 4/nuclear factor kappa B–pathway related proteins are affected in living donor renal transplant recipients.

The response to opportunistic infections including BK polyomavirus and cytomegalovirus is largely mediated by peripheral blood mononuclear cells.[22-24] Samples from patients with cytomegalovirus infection show higher expression of immunoglobulin-like receptor B1 in peripheral blood mononuclear cells compared with those who do[22]; therefore, cytomegalovirus may cause modulation of patients' innate and adaptive immune systems.[22] Kumar *et al.*[23] quantified interferon gamma production from peripheral blood cells after cytomegalovirus-specific stimulation to demonstrate the feasibility of early discontinuation of antivirals.[23] Leboeuf *et al.*[24] measured the release of interferon gamma after stimulation of peripheral blood mononuclear cells from patients with virus-specific peptides to predict clearance of BK polyomavirus in renal transplant recipients. Recently Hudson *et al.*[11] showed the major importance of long noncoding RNA for reactivation after viral infection. By performing RNA sequencing they showed that long noncoding RNAs are sufficient to define naive, effector, and memory $CD8^+$ T-cell subsets after viral infection. These memory T cells show greatly increased kinetics to protect the host from reinfection.[11] Long noncoding RNAs affect cells by binding to their transcription factors and modulating their activity and gene expression.[25] Hence, a lower MGAT3-AS1/beta-actin ratio may indicate higher susceptibility to virus reactivation, although the exact mechanisms are yet unknown.

We calculated the median from repeated MGAT3-AS1/beta-actin ratio measurements within the first

postoperative month, because it is well known that both the intensity and the duration of immunosuppression may support reactivation of virus. Post-transplant viremia of BK polyomavirus and cytomegalovirus is usually observed after the first postoperative month because reactivation of virus replications is assumed to be associated with immunosuppressive therapies.[3,4] Most infections after renal transplantation progress through well-characterized stages.[1–3] Reactivation of BK polyomavirus precedes tubulointerstitial nephritis and loss of kidney function in renal transplant recipients.[8,9] In patients with viremia of BK polyomavirus an established antiviral treatment is currently not available. Current guidelines suggest to discontinue antiproliferative mycophenolate and to reduce the calcineurin inhibitor dose to control the replication of BK polyomavirus. Patients with viremia of cytomegalovirus may progress to enteritis, pneumonitis, retinitis, and nephritis, leading to increased risk of allograft failure and death.[5–7] In patients with viremia of cytomegalovirus, antiviral treatment may be limited by drug-related adverse events, including leukopenia, thrombocytopenia, and diarrhea. To minimize both the risks for viremia and of treatments, a simple test to determine an increased risk for viremia is preferable.

We observed that the duration of dialysis before transplantation was significantly longer in renal transplant recipients in whom viremia developed, which is in accordance with the findings of Bal *et al.*,[26] who showed that longer dialysis vintage was a risk factor for cytomegalovirus reactivation after transplantation. We observed combined BK polyoma virus and cytomegalovirus reactivation, which is in accordance with findings of Blazquez-Navarro *et al.*,[27] who showed that 57 of 540 kidney transplant recipients (11%) had combined virus reactivation. In our study, all living donor renal transplant recipients received prophylaxis with either acyclovir or valganciclovir, and we observed viremia of cytomegalovirus in 7% of recipients within 3 months after transplantation. That finding is in agreement with results reported by Luan *et al.*,[6] who observed viremia of cytomegalovirus in 10% of recipients with valganciclovir prophylaxis within 6 months after transplantation.

Our study supports earlier observations indicating that some immunosuppressive regimes do seem to increase the reactivation of virus replication.[7] We showed that patients with viremia were more likely to have induction therapy with rituximab, and prednisolone, thymoglobulin, or both. DeKeyzer *et al.*[7] reported that induction therapy with thymoglobulin is associated with a 2- to 5-fold increase of viremia. Our study now adds that, in particular, those recipients receiving these induction regimes and who had lower MGAT3-AS1/beta-actin ratios show an increased risk for viremia. Taken together, a lower MGAT3-AS1/beta-actin ratio indicates an increased risk for viremia of BK polyomavirus and cytomegalovirus in living donor renal transplant recipients.

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

SN and MT conceived and designed the study. SN and MR performed the measurements. SN, MR, SVH, and MT performed the statistical analysis and interpreted the data. SN, MR, SVH, and MT wrote the draft of the report. All authors revised the report for important intellectual content.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

STROBE Checklist.

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