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The detection of BKPyV genotypes II and IV after renal transplantation as a simple tool for risk assessment for PyVAN and transplant outcome already at early stages of BKPyV reactivation

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ABSTRACT

Background: After reactivation the BK-polyomavirus (BKPyV) associated nephropathy (PyVAN) is observed in 1–10% of renal transplant recipients, of which up to 80% undergo graft failure. BKPyV reactivation after renal transplantation was associated with donor-derived serotypes against which the recipient has no immunological protection. However, PyVAN risk assessment seroactivity testing is a time-consuming and cost intensive process.
Objectives: Since BKPyV serotypes can be attributed to distinct genotypes I to IV, in the present study we retrospectively analyzed whether a simple PCR-based BKPyV genotyping assay might be a fast and inexpensive method to assess the risk for PyVAN and transplant outcome already at early stages of BKPyV reactivation.
Study design: 56 patients who were renal transplanted and tested positive for BKPyV viremia were included into the study. The BKPyV-VP1-coding sequences were PCR-amplified, sequenced, and subjected to genotyping. For group specific analysis patients were grouped in genotype I (n = 46) and a second group including genotype II and IV (n = 10) and associated with their clinical outcomes.
Results: The most abundant genotype I was detected in 46 of 56 (82%) patients, however, in the genotype II and IV group PyVAN was twice as frequent as compared to the genotype I group 24 months after transplantation (8 of 10 (80%) vs. 17 of 46 (37%); p = 0.001). Accordingly, graft failure was significantly more frequent in the genotype II and IV group (3 of 10 (30%) vs. 2 of 46 (4%); p = 0.007).
Conclusion: PCR-based BKPyV genotyping might represent a fast and inexpensive method to assess the risk for PyVAN and transplant outcome already at early stages of BKPyV reactivation even if matched samples of the donor are not available.

1. Background

The *human polyomavirus type 1* (BKPyV) belongs to the genus *Polyomavirus* of the family of non-enveloped *Polyomaviridae* and possess an approx. 5 kb small circular double-stranded DNA genome. The BKPyV associated nephropathy (PyVAN), which occurs in 1–10% of renal transplant recipients, is a substantial cause of premature renal transplant failure [1–3]. The PyVAN is stratified into three groups (A, B and C) according to the histological detected interstitial inflammation, tubular atrophy and fibrosis. Depending on the PyVAN stage, the risk of graft failure differs from < 10% in patients with PyVAN A to > 80% in patients with PyVAN C [4].

Primary infection with BKPyV occurs mainly in the first decade of life as evidenced by BKPyV seroprevalence of up to 90% in adults [5,6]. So far, no highly effective antivirals for the treatment of BKPyV are available. Controversially, cidofovir was supposed to limit BKPyV

Abbreviations: BKPyV, BK-polyomavirus; DNA, deoxyribonucleic acid; GT, genotype; KDIGO, Kidney Disease Improving Global Outcomes (organization); Ml, milliliter; NAb, neutralization antibody; PCR, polymerase chain reaction; PyVAN, polyomavirus associated nephropathy; VP1, viral capsid protein 1

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replication, however the effect was limited and the severe nephrotoxic side effects may in addition to the BKPyV infection impair the renal function [7–9]. The lipid-conjugated cidofovir derivate brincidofovir (CMX001) has an enhanced oral bioavailability and a higher antiviral activity *in vitro,* however, the *in vitro* results have not been validated in clinical studies [10,11]. Moreover, the immunosuppressive agent leflunomide, which is commonly used for the treatment of rheumatoid arthritis, was proven to inhibit BKPyV replication *in vitro* but a randomized study of leflunomide as a treatment for PyVAN did not reveal beneficial effects on graft survival [10,12,13]. An alternative treatment is based on immunoglobulin mediated prevention of cellular infection achieved by neutralizing antibodies [14,15]. However, well designed clinical trials are still missing to prove the therapeutic efficacy of immunoglobulin treatment in the management of BKPyV infection [10]. Thus, the only remaining treatment option is the reduction of the immunosuppression and subsequent immunological re-control. However, this preemptive strategy does not fully eliminate the risk of PyVAN [16,17] and a reduction in immunosuppression increases the risk of graft rejection.

Immunosuppressive therapy based on tacrolimus-mycophenolic acid, low or absent BKPyV-specific T cell responses after kidney transplantation as well as donor neutralizing serostatus are considerable risk factors for BKPyV reactivation [6,18,19]. In addition, rearrangements in the promoter region of the virus (non-coding control region; NCCR) have been described as additional risk factors. In this respect rearrangements seem to appear more frequent in patients with PyVAN and are associated with altered promoter activity [20,21].

Importantly, genotype mismatch between recipients neutralization profiles before transplantation and their subsequently replicating strain after transplantation was recently discovered as an important risk factor for BKPyV viremia [22]. The four distinct BKPyV genotypes (GT) I–IV have been previously described, while GT I and IV can be further subdivided in subgenotypes Ia, Ib1, Ib2, Ic as well as IVb1 and IVc2 [23,24]. A cross-neutralizing antibody study could demonstrate that GTs I to IV reflect distinct serotypes. In addition, GTs Ib1 and Ib2 act as distinct serotypes while both subtypes of GT IV are serologically cross reactive [24]. The BKPyV GTs were proposed to exert distinct biological properties as cell tropism and some genotypes supposed to be more frequently associated with disease and *in vitro* cytopathic effects [25]. Recipients developing weak BKPyV GT-specific neutralizing antibody responses against donor-derived strains have a significantly higher risk of developing PyVAN. Thus, BKPyV GT-specific neutralizing antibody titres might be used for risk prediction [22,26].

Polymerase chain reaction (PCR) is currently the preferred method to identify patients with BKPyV replication [4]. While patients with high-level BKPyV DNA (> 10.000 copies/ml) are frequently associated with PyVAN, it has been shown that low level viremia has no impact on kidney function and graft loss [27,28]. Thus, it is highly recommended to implement regular follow up examinations with screening for BKPyV replication in serum after renal transplantation [28–30].

2. Objectives

Since the screening for GT-specific neutralizing antibodies requires high laboratory expertise and time expenses, we evaluated whether PCR-based BKPyV genotyping already at early stages of BKPyV reactivation might represent a fast and inexpensive method for PyVAN risk evaluation also in low-level BKPyV-DNA positive patients even if matched samples of the donor are not available.

3. Study design

In this retrospective cross-sectional single center study we analyzed 56 renal transplant recipients, who were tested positive for BKPyV viremia between January 2015 and October 2016. Patients were transplanted between 2008 and 2016. Inclusion criteria were a minimum age of 18 years at the time of transplantation.

After transplantation, all patients received regular follow up examinations at the department of Nephrology (University Hospital Essen). As a marker for viral replication, screening for BKPyV DNA in serum was performed at 1, 3, 6, 12, and 24 months and yearly after transplantation. The screening protocol was modified based on recommendation of the KDIGO guidelines 2009 [31]. In addition to the scheduled BKPyV screening, patients were tested for BKPyV DNA in serum when allograft dysfunction was detected. Graft biopsy was indicated in case of graft dysfunction or viral persistence. All samples were analyzed via quantitative real-time PCR during routine diagnostics as described elsewhere [32]. Serum samples from patients tested positive for BKPyV replication were collected at the Institute for Virology (University Hospital Essen). In addition, biopsy samples of patients with biopsy proven PyVAN were obtained from the Institute of Pathology of the Medical School Hanover. For BKPyV genotyping, DNA was obtained from serum or biopsy samples of BKPyV positive patients and the VP1 coding region was sequenced. Patients were grouped depending on the VP1 related GT. The first group includes patients with GT I only (GT I) while the second group comprises patients with GTs II and IV. In agreement with the low prevalence, we did not detect GT III in our cohort. The study was approved by the Ethic committee of the University Hospital Essen (14-6028-BO). Data obtained included demographics, transplant characteristics, BKPyV viral load as well as recipient and donor data. Data concerning intermediate transplant outcome like transplant function, graft failure and PyVAN diagnosis was obtained within 24 months after transplantation.

4. VP1 amplification and analysis from serum and biopsy samples

In samples tested positive for BKPyV, DNA was extracted from 400 µl plasma or biopsy samples using QIAmp®; DNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. A semi-nested PCR approach was used with the outer primer pairs BK-VP1-1fw/BK-VP1-rv, and the inner primer pair BK-VP1-2fw/BK-VP1-rv (BKV-VP1-1fw CCCGTGCAAGTGCCAAAACTAC; BKV-VP1-2fw CAAGTGCCAAAA CTACTAATAAAAGG; BKV-VP1-rv CATGCTCATGCACTTTTTGTGACC) covering nucleotide positions 1624–1981 in the BKPyV (Dunlop) genome (Genbank accession number V01108). These primers show efficiency against all genotypes [33].

All amplicons were analyzed by electrophoresis in 1.5% agarose gels and visualized using ethidium bromide staining. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) and subjected to Sanger sequencing. Genotyping was performed as described before [34].

5. Statistics

Depending on initial normality distribution testing unpaired t-test, Mann-Whitney U test and Fisher's exact test were used as applicable. Statistical analysis was performed with GraphPad Prism (V 5.01).

6. Results

6.1. BKPyV genotyping and genotype distribution

BKPyV genotyping from serum and biopsy samples could be performed based on 56 patient derived VP1-sequences (42 patients from serum samples, in 5 patients from biopsy samples and in 9 patients from both (serum and biopsy samples)). GT I was detected in 46 of 56 (82%) patients. GT II was detected in 2 (4%) and GT IV was detected in 8 of 56 (14%) patients. GT III could not be detected in this cohort (Fig. 1; Table 1). All 9 patients with genotype detection from serum and biopsy samples had the same genotype detected. Neither there was a difference of renal transplant recipients and donors between the GT I, GT II and IV group in gender, age or type of donation (cadaver vs. living) nor rest

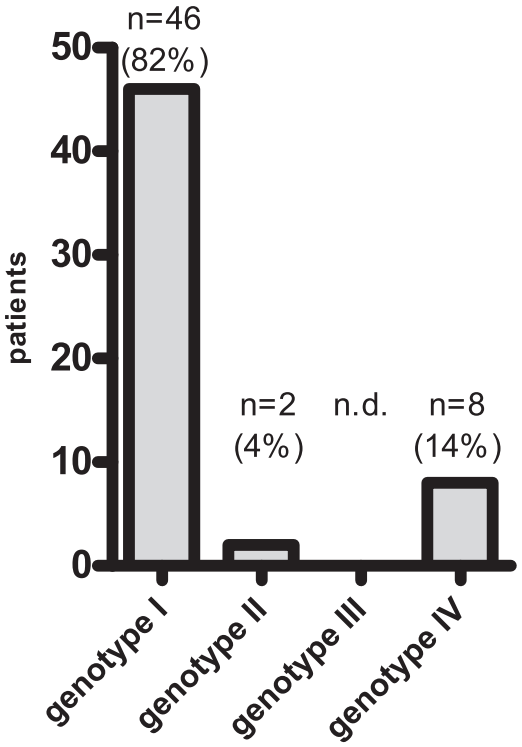

Fig. 1. BKPyV genotype distribution of 56 BKPyV positive renal transplant recipients from the study. Amplification and genotyping was performed from serum and biopsy samples as available. Genotype I was detected in 46 of 56 (82%) patients. Genotype II was detected in 2 (4%) and genotype IV was detected in 8 of 56 (14%) patients. Genotype III could not be detected in this cohort. n.d. no data.

diuresis, cold and warm ischemia time, and occurrence of first viremia (Table 1). All patients were treated with a corticosteroid containing immunosuppressive regimen after transplantation. Both groups were treated mainly with tacrolimus based immunosuppression (GT I group 93%; GT II and IV group 90%) and IL-2 receptor antibody as induction therapy (GT I group 93%; GT II and IV group 90%). In the GT I group two of 46 (5%) were treated with cyclosporine A and five patients (11%) were treated with a regimen including mTOR inhibitos (sirolimus (n = 1) and everolimus (n = 4)). In the GT II and IV group one patient (10%) was treated with cyclosporine A instead of tacrolimus and three patients (30%) were treated with a regimen including mTOR inhibitos (everolimus (n = 3)). There was no difference in cellular and humoral rejection between the groups within 24 months after transplantation. The transplant function one month after transplantation was the same in both groups (44.6 +/- 13.8 vs. 44.1 +/- 6.2; Table 2). In addition, no significant difference in graft failure censored transplant function was observed at 12 and 24 months after transplantation (12 months 41.6 +/- 14.9 vs. 36.1 +/- 15.3, p = 0.35; 24 months 39.4 +/- 16.1 vs. 34.3 +/- 12.8, p = 0.43).

In comparison to patients in the GT I group patients in the GT II and IV group had significantly higher maximal detected serum viral loads after renal transplantation (2.3E7 +/- 5.1E7 vs. 7.0E6 +/- 1.8E7; p = 0.03; Fig. 2c).

To investigate whether the significantly higher viral load of the group with rare GTs also leads to clinical complications, we compared the intermediate clinical outcome of the patients. 26 of 56 (45%) patients were biopsy proven diagnosed with PyVAN within 24 months after transplantation. PyVAN within 24 months after transplantation was significantly more frequent in the GT II and IV group in comparison to the GT I group (8 of 10 (80%) vs. 17 of 46 (37%); p = 0.001; Fig. 2A and Table 2). In addition, graft failure within 24 months after transplantation was significantly more frequent in the GT II and IV group (3 of 10 (30%) vs. 2 of 46 (4%); p = 0.007; Fig. 2B). This data indicate that genotyping is highly predictive for clinical outcome. Thus, preventive genotyping might be used for time and cost inexpensive risk assessment.

7. Discussion

In this retrospective study, we analyzed the clinical impact of rare BKPyV GTs (GT II and IV) in comparison to the most frequent BKPyV GT I on the intermediate renal transplant outcome. In 56 adult patients tested positive for BKPyV viremia after renal transplantation the BKPyV VP1 GT was analyzed and correlated with the clinical transplant outcome. Amplification and genotyping was successful in patients with a serum viral load higher $10E^3$ copies/ml.

The population of our study represents a reliable cohort since GT I was the most frequent detected GT (n = 46; 82%) in the cohort of renal transplant patients. To our current knowledge, this cohort describes the BKPyV genotype distribution with the greatest samples size from serum

Table 1
Patients characteristics.

	genotype I n = 46	genotype II and IV n = 10	*p*	statistical method
recipient male sex, n (%)	30 (65%)	6 (60%)	0.73	f
recipient mean age (SD), y	52.4 (12.3)	57.5 (13.3)	0.38	mw
cadaver, n (%)	36 (78%)	9 (90%)	0.66	f
donor male sex, n (%)	23 (51%)	6 (60%)	0.73	f
donor mean age (SD), y	53.4 (15.5)	59.4 (10.3)	0.27	mw
rest diuresis, ml (SD)	720.7 (809.2)	588.9 (555.5)	0.78	mw
cold ischemia, hours (SD)	9.6 (5.8)	12.6 (7.2)	0.21	mw
warm ischemia, minutes (SD)	26.8 (9.2)	28.3 (7.8)	0.44	mw
cellular rejection n (%), within 24 months after Rtx	18 (39%)	3 (30%)	0.72	f
humoral rejection n (%), within 24 months after Rtx	2 (4%)	0 (0%)	1.0	f
induction therapy ATG, n (%)	3 (7%)	1 (10%)	0.53	f
induction therapy IL2-R-AB, n (%)	43 (93%)	9 (90%)		
tacrolimus based immunosuppression, n (%)	43 (93%)	9 (90%)	0.55	f
corticosteroid containing regimen, n (%)	46 (100%)	10 (100%)	1.0	f
mycophenolic acid containing regimen, n (%)	42 (91%)	7 (70%)	0.1	f
m-TOR inhibitor containing regimen, n (%)	5 (11%)	3 (30%)	0.14	f
occurrence of first viremia in months (SD)	18.7 (28.0)	9.7 (12.8)	0.26	lr
death within 24 months after Rtx	2 (4%)	1 (10%)	0.45	f

Rtx renal transplantation; f fisher's exact test; mw mann whitney U test; ut unpared t-test; lr logrank test.

Table 2
Graft outcome.

	genotype I n = 46	genotype II and IV n = 10	*p*	statistical method
eGFR at 1 months (mL/min/1.73 m2) (SD)	44.6 (13.8)	44.1 (6.2)	0.9	ut
eGFR at 12 months (mL/min/1.73 m2) (SD)	41.6 (14.9)	36.1 (15.3)	0.35	ut
eGFR at 24 months (mL/min/1.73 m2) (SD)	39.4 (16.1)	34.3 (12.8)	0.43	ut
graft failure within 24 months after Rtx, n (%)	2 (4%)	3 (30%)	**0.007**	lr
biopsy proven PyVAN within 24 months after Rtx, n (%)	17 (37%)	8 (80%)	**0.001**	lr
highest detected viral load, median (SD)	7.02E + 06 (1.8E + 07)	2.3E + 07 (5.1E + 07)	**0.03**	mw

Rtx renal transplantation; f fisher's exact test; mw mann whitney U test; ut unpared t-test; lr logrank test.

and or biopsy isolates since most studies with genotype analysis performed genotype amplification from urine samples and lower samples sizes [35–37]. GT IV (n = 8; 14%) was the second most detected GT. GT II was detected twice (4%) and GT III was not detected in this cohort. These findings correspond with the normal GT distribution in the human population worldwide [35,36]. The most important factors for worse transplant outcome like rejection rates, age of recipient and donor, type of donation, and cold and warm ischemia time did not differ between the groups [38–40] (Table 1). Neither did risk factors affecting BKPyV viremia after renal transplantation like type of immunosuppression and induction therapy differ between the groups [41,42]. Therefore the impact of rare GTs on the intermediate transplant outcome can be highlighted in this cohort.

The PyVAN diagnosis within 24 months after transplantation was significantly higher in patients with rare GTs (p = 0.001; Fig. 2A). In addition, patients with rare BKPyV GTs experienced significantly more frequent graft failures within 24 months after transplantation in comparison to patients with the common BKPyV GT I (p = 0.007; Fig. 2B). Thus, genotyping represents a rapid and cost-effective method to assess PyVAN risk even in the early stages of reactivation with low viremia and may prevent the progress of early PyVAN stages by preemptive reduction of immunosuppressive therapy.

Pastrana and colleagues demonstrated that BKPyV GTs I, II, III, and IV are fully distinct serotypes [24]. The authors performed neutralization assays on sera from 50 healthy human subjects. While nearly all healthy subjects had BKPyV GT I neutralizing antibodies, a majority of subjects did not detectably neutralize GT III or IV. The authors concluded that individuals who were infected with one BKPyV serotype may remain humorally vulnerable to other BKPyV serotypes after implementation of T-cell immunosuppression. This factor is essential because BKPyV infection after renal transplantation is mostly donor derived [43]. Furthermore, renal transplant recipients lacking neutralization antibody titers (NAb) against BKPyV seem to be at higher risk for BKPyV replication after renal transplantation [26]. As recently described by Solis and colleagues the GT specific immunity has a great impact on the reactivation of BKPyV after renal transplantation [22]. The authors prospectively analyzed NAb of 168 renal transplant recipients and 69 donors before and after transplantation. The majority of patients were positive for GT I antibodies before transplantation. These findings are in agreement with the detected NAb serotype distribution. Since delayed and weak NAb response significantly increased BKPyV viremia and PyVAN risk after transplantation and the authors concluded that BKPyV genotype-specific neutralizing antibody titers may serve as a new predictive marker for PyVAN risk evaluation.

Solis and colleagues performed VP1 based genotyping in patients with BKPyV replication and found that replicating strains were consistent with donor transmission in 95% of cases of early BKPyV replication. Furthermore, GT mismatch between the recipient and donor significantly increased the viremia risk [22]. However, in this study a genotype specific association with PyVAN was not described by the authors, which was most likely due to the small serum sample size (n = 28). Importantly, since PyVAN emergence was exclusively observed in patients with high viremia, serum represent the most relevant specimen for BKPyV genotyping used for risk evaluation.

Consequently, in the current study PyVAN emergence and graft failure detection within 24 months after transplantation in patients with rare GT might most likely reflect low or missing immunologic control against the circulating strain and could be crucial for the significant higher rates of increased replication of rare GTs.

Another important influence on the worse outcome in patients with GT II and IV detection may be based on different BKPyV GT specific cytopathic effects. In particular, more severe cytopathic effects of *in vitro* BKPyV GT IV infected Vero cells were observed 26 days post infection, even though the replication level was the same as of GT I infected cells [44]. Thus, GT II and IV BKPyV reactivation might result in more severe PyVAN progress with higher rates of inflammation, atrophy and fibrosis. In the current study the maximal detected viral load was significantly higher in patients with rare GTs (Fig. 2c; p = 0.03), although both groups exceeded the detection limit of 10.000 copies/ml that is associated with PyVAN emergence [27,28].

As a further risk factor, rearrangements in the non-coding control region (NCCR) were described. These significantly affect the NCCR-promoter activity and have been more frequently observed in patients with PyVAN [20,21]. However, since rearrangements are diverse and their impact on viral replication is not predictable, NCCR-genotyping in terms of simple risk stratification remains unsuitable.

In the context of missing an effective antiviral treatment for BKPyV and early identification of patients with high risk for PyVAN emergence, donor and recipient GT specific seroactivity and BKPyV genotyping in case of BKPyV replication seem to be an important tool for accomplishing PyVAN risk evaluation. Thus, we suggest that PCR-mediated genotyping should be routinely performed to identify risk groups at an early stage, which is a feasible and low cost standard laboratory method, available in most diagnostic institutes. Consequently, patients with rare BKPyV replicating strains might benefit from early reduction of immunosuppression and prophylactic immunoglobulin administration [17] until recovering GT specific immune control.

Limitations of this study are its retrospective character and its small samples size, however, since the genotype distribution matches the distribution of the known literature the analyzed cohort is representative. All patients from both groups (GT-I and GT II and IV) exceeded the limit of 10.000 BKPyV copies/mL. Furthermore, BKPyV viremia risk factors and important graft outcome parameters did not differ between groups.

8. Conclusion

The detection of rare BKPyV genotypes (II and IV) is associated with higher rates of PyVAN diagnosis and worse intermediate transplant outcome after renal transplantation. Therefore, BKPyV genotyping seems to be an additional fast and inexpensive method to determine the risk for PyVAN and transplant outcome already at early stages of BKPyV reactivation.

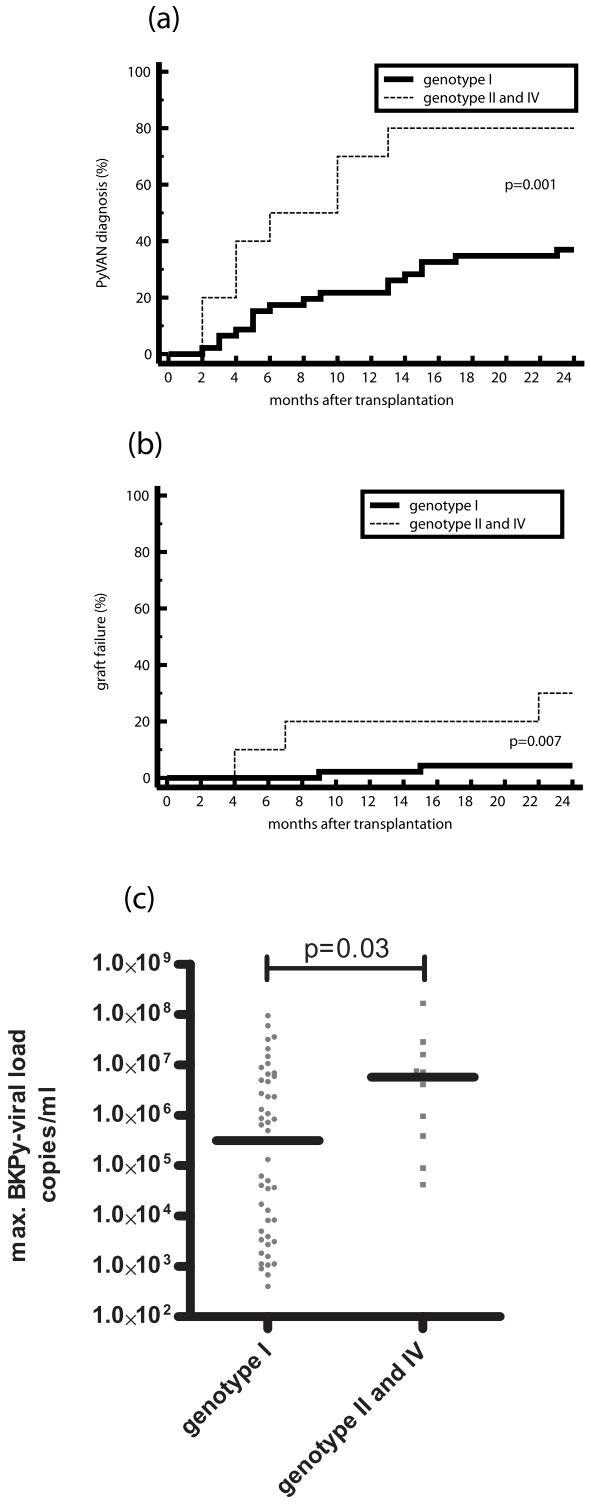

Fig. 2. a + b: Clinical and virological outcome of BKPyV positive renal transplant recipients. Kaplan-Meier-curve for PyVAN diagnosis (A) and graft failure (B) within 24 months after renal transplantation of patients with BKPyV viremia stratified by BKPyV genotype detection (genotype I vs. genotype II and IV group). Fig. 2c Maximal detected viral load in patients after renal transplantation. The groups were stratified by BKPyV genotype detection (genotype I vs. genotype II and IV group).

Authors' contributions

JK and MW conceived, designed, and performed the study, performed the assays, collected and analyzed the data, and wrote the manuscript; SC, BW, OEA, AB and OW analyzed the data, and helped to draft the manuscript; JHB and UL performed biopsy analysis and DNA amplification and helped to draft the manuscript. UD, AK and JV analyzed the data and participated in its design and coordination.

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Conflict of interest

OW has received grants for clinical studies, speaker's fee, honoraria and travel expenses from Amgen, Alexion, Astellas, Basilea, Biotest, Bristol-Myers-Squibb, Correvio, Chiesi, Gilead, Hexal, Janssen, Dr. F. Köhler Chemie, MSD, Novartis, Roche, Pfizer, Sanofi and TEVA. The other authors declared no conflict of interests.

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