Risk factors for Epstein–Barr virus reactivation after renal transplantation: Results of a large, multi-centre study

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SUMMARY

Epstein–Barr virus (EBV) reactivation is a very common and potentially lethal complication of renal transplantation. However, its risk factors and effects on transplant outcome are not well known. Here, we have analysed a large, multi-centre cohort (N=512) in which 18.4% of the patients experienced EBV reactivation during the first post-transplant year. The patients were characterized pre-transplant and two weeks post-transplant by a multi-level biomarker panel. EBV reactivation was episodic for most patients, only 12 patients showed prolonged viraemia for over four months. Pre-transplant EBV shedding and male sex were associated with significantly increased incidence of post-transplant EBV reactivation. Importantly, we also identified a significant association of post-transplant EBV with acute rejection and with decreased haemoglobin levels. No further severe complications associated with EBV, either episodic or chronic, could be detected. Our data suggest that despite relatively frequent EBV reactivation, it had no association with serious complications during the first post-transplantation year. EBV shedding prior to transplantation could be employed as biomarkers for personalized immunosuppressive therapy. In summary, our results support the employed immunosuppressive regimes as relatively safe with regard to EBV. However, long-term studies are paramount to support these conclusions.

Key words

EBV < Infection, Outcome < Kidney Clinical, Complications < Kidney Clinical, PTLD < Malignancies and long-term complications
Introduction

Infections with viral pathogens can result in major complications after solid organ transplantation. Immunosuppression can disrupt the control of latent infections in organ recipients and increase the risk of transmission from the donor or the general population, leading to life-threatening viral infections and reactivations [1,2]. One of the most frequently occurring viral pathogen in renal transplantation is the Epstein–Barr virus (EBV) [3,4]. Approximately 90% of the population are latently infected with EBV in high- and middle-income countries [5–7]. After transplantation, EBV primary infections and reactivations posit a serious risk known as post-transplant lymphoproliferative disorders (PTLD), ranging from asymptomatic self-contained viraemia, to potential lethal, malignant disease [8,9].

In our previously published work derived from the Harmony cohort, we found a 20% prevalence of EBV viraemia either pre-transplant or during the first post-transplant year [3,4]. The main risk factors for EBV viraemia were found to be rabbit antithymocyte globulin (rATG) induction treatment, as well as EBV mismatch (donor seropositive, recipient seronegative – D+/R−) and cytomegalovirus D+/R− mismatch [3,4]. However, several aspects of the EBV viraemia course are still unclear. For example, the relationship between pre-transplant and post-transplant viraemia has not been sufficiently studied. Importantly, no reliable models for the early prediction of EBV reactivation to assist therapeutic decisions currently exist [10].

In this study, we perform a further and more detailed characterization of EBV reactivation within our well-characterized Harmony cohort [3,4]. Here, we provide evidence on the significance of pre-transplant EBV viral load for the transplant course as well as risk factors for EBV reactivation potentially enabling personalization of immunosuppressive therapy. Furthermore, we offer evidence that the employed immunosuppressive regimes were relatively safe regarding severe EBV-associated complications.

Methods

Patient population and medication

We have conducted a sub-study within the randomized, multi-centre, investigator-initiated Harmony trial (NCT 00724022) to determine the risk factors and impact of EBV reactivation [4,11]. Thus, the patients were characterized for EBV reactivation and clinical markers pre-transplant and at seven post-transplant visits [4]. The study was carried out in compliance with the Declaration of Helsinki and Good Clinical Practice.

As described previously, the patient cohort received one of three immunosuppressive therapy regimes [11]. Thus, patients received an induction therapy with basiliximab or rATG. Maintenance therapy consisted of
tacrolimus, mycophenolate mofetil (MMF) and corticosteroids, whereas for some patients, corticosteroids were withdrawn at day 8. According to the study protocol, patients with D+/R- mismatch for either CMV or EBV as well as all patients with rATG induction, received a valganciclovir (VGCV) prophylaxis [11]. VGCV prophylaxis was defined as VGCV treatment initiated during the first 14 days, as explained before [12]. Reported MMF dose and tacrolimus trough levels correspond to this same 14 day threshold.

Monitoring of viral load

Patients were monitored for transplant outcomes during the first post-transplant year. Thus, peripheral blood samples were centrally monitored for EBV, cytomegalovirus (CMV) and BK virus (BKV) by TaqMan qPCR along eight visits, as described previously [4]. Shortly, blood samples were taken at day 0 (pre-transplant, pre-Tx), 2nd week (2w), 1st month, 2nd month, 3rd month, 6th month, 9th month and 12th month post-transplant. DNA was isolated from whole blood samples, for EBV and CMV, or serum, for BKV, following the manufacturer’s instructions. The qPCR was performed employing the Prism 7700 Sequence Detector. The detection level was determined as the lowest viral load measured within the range of linearity (250 copies/ml)[4]

Duration of EBV viraemia was calculated as the time between the first and the last post-transplant samples with detectable EBV load, including possible episodes of negative viral load in-between. Patients with an EBV duration over 120 days were classified as EBV<sub>chron</sub>. Based on their peak viral load values for EBV, the patients were classified as follows: patients with at least one viral load measurement over detection limit were classified as EBV<sup>+</sup>; patients with at least one viral load measurement over 1000 copies/ml were classified as EBV<sup>increased</sup>; patients with no viral load measurement over detection limit were classified as EBV<sup>−</sup>; lastly, patients with EBV viral load over detection limit at pre-Tx were classified as pre-Tx-EBV<sup>+</sup>.

Monitoring of transplant outcomes

The patient cohort was monitored at the local centres at the eight pre-defined visits. Thus, estimated glomerular filtration rate (eGFR), as well as full blood count and routine chemistry tests were estimated. eGFR was assessed using the CKD-EPI formula, measured in ml/min/1.73 m<sup>2</sup> [13]. Suspected episodes of acute rejection were confirmed through biopsy; histologic characteristics were described according to the Banff criteria of 2005 [4,11,14].

Characterization of marker subsets for EBV viraemia

The patients were characterized for a multi-level biomarker panel consisting of five subsets, at pre-Tx and 2w. Shortly, gene expression markers were measured employing TaqMan Gene Expression Assays (Thermo Fisher Scientific). Leucocyte subsets were determined based on the epigenetic quantification, as described before [15,16]. Screening of serum antibody binding profile (SAB) was performed employing HLA-1 mixed antigen bead assay; the raw mean fluorescence intensity for each bead was employed for prediction [17]. The urinary metabolomics spectrum was measured by nuclear magnetic resonance (NMR) spectroscopy at numares AG (Regensburg, Germany). Finally, clinical data were measured de-centrally and were provided by the transplantation centres. For more details on the methods and the list of measured markers, see the Supplementary Methods.

Statistical analysis

Prediction of EBV reactivation and identification of risk factors were performed for each of the five pre-defined marker subsets, employing data from pre-Tx and 2w. Here, EBV<sup>+</sup> patients were compared with EBV<sup>−</sup> and EBV<sup>increased</sup> with EBV<sup>−</sup>. Patients with detectable EBV load during or before sampling were disregarded for the analysis, as we aimed for prognostic models and risk factors.

Prediction of EBV viraemia was performed employing the machine learning algorithm random forest for classification (R package randomForest, version 4.6-14) with cross-validation [18]. The performance of the EBV classification was assessed by the balanced accuracy (BACC).

For the analysis of individual EBV risk factors, we did not include the SAB and the urine metabolome subset, since these are to be interpreted as a whole rather than as individual markers. Markers demonstrating a significant difference between the viraemia sub-cohort and the EBV<sup>−</sup> were considered as potential EBV prognostic markers. The significance of this difference was tested using the two-tailed Mann–Whitney test for quantitative factors and two-tailed Fisher’s exact test for categorical factors. Adjusted P values were calculated employing the Benjamin & Hochberg method.

Categorical variables are summarized here as numbers and frequencies; quantitative variables are reported as median and interquartile range (IQR). The significance of an association with EBV reactivation was tested
employing Mann–Whitney or Fisher test, as for risk factors. Correlations were evaluated employing Spearman’s rank correlation. Forest plots depict the odds ratio (OR) as square point and 95% confidence interval (95% CI) as a line. For more details on the statistical methods, see the Supplementary Methods.

Results

Identification of EBV risk factors

Characteristics of EBV reactivation in the cohort

A total of 512 patients were characterized for EBV viraemia: 94 patients (18.4%; hereafter EBV+ group) had post-transplant EBV viral load, 44 patients (46.8%) of them had clinically relevant viral loads of over 1000 copies/ml in blood (from now on EBVincreased). Patients who did not suffer from detectable EBV reactivation during the first post-transplant year are here referred to as EBV−.

For most patients, viraemia was only episodic. 12 patients suffered from prolonged reactivations of over 4 months (from now on EBVchron). These EBVchron patients suffered from particularly severe EBV reactivations, with significantly higher peak viral loads compared with the rest of the EBV+ patients (3198 [1194–6862] vs. 791 [384–2440] copies/ml; P = 0.010). In fact, there was a highly significant correlation between reactivation duration and peak viral load (r = 0.37; P < 0.001).

Pre-transplant EBV load is a risk factor for post-transplant EBV reactivation

A total of 373 (72.9%) patients of the cohort were characterized for their pre-transplant EBV load. A substantial number of patients (6.1%, N = 23; hereafter pre-Tx-EBV+) showed detectable viral load in blood before transplant, with a median load of 1064 [810–2764] copies/ml. As expected, pre-Tx-EBV+ patients had a significantly higher incidence of EBV reactivation post-transplant (OR: 4.89 [1.85–12.79], P = 0.001). While these patients also had a tendency towards higher viral loads, the difference was not significant (1765 [602–4097] vs. 782 [384–2440] copies/ml; P = 0.222). For the duration of reactivation, we found a borderline significant tendency towards longer reactivation among pre-Tx-EBV+ patients (14 [0–110] vs. 0 [0–12] days; P = 0.082). Pre-Tx-EBV+ was not significantly associated with EBVchron (OR: 2.53 [0.35–14.42]; P = 0.351).

No effective multi-parameter predictive model of EBV reactivation could be identified

Here, we analysed a large number of markers (see Supplementary Methods) at pre-Tx and 2w, with the goal of predicting which patients become are EBV+ or EBVincreased. First, we analysed the marker subsets as a whole, with the goal of determining whether certain marker types are more adequate for prediction of EBV viraemia. However, none of the marker types was able to predict EBV reactivation satisfactorily (Fig. S1): The best performing marker subset was the SAB profiles at pre-Tx (BACC = 61.1% sensitivity = 33.3%, specificity = 88.9%).

Immunotolerance marker genes, male sex and metabolic alterations were prognostic for EBV reactivation

As no effective multi-parameter predictive model could be generated, we evaluated whether any potential markers for EBV reactivation could be identified. Previously, an association of EBV+ with rATG induction and of EBVincreased with D+/R− mismatch for CMV and EBV had been observed [4]. In addition to the previous results, we observed an association of EBV+ with male sex (OR: 1.71 [1.02–2.94]; P = 0.033; Figs 1 and S2).

Figure 1 Forest plot comparing EBV+ versus EBV− patients for selected demographic factors. The square denotes the odds ratio, whereas an odds ratio over 1 means an increased higher incidence among EBV+ patients; the grey lines denote the 95% confidence interval.
Furthermore, we evaluated our multi-level marker panel to identify potential markers predictive of EBV reactivation (Tables S1 and S2). Only the expression of the genes CD200 and LAG3 – immunological co-inhibitory – at 2w was potentially associated with EBV reactivations (Fig. S3). However, the encountered differences for these genes were not significant when adjusting for multiple testing; none of the measured markers showed a significant association with EBV reactivation after adjustment (see Tables S1 and S2).

Evaluation of the impact of EBV reactivation on post-transplantation

*Post-transplant EBV reactivation was associated with higher incidence of acute rejection*

We examined the transplant outcome of EBV+ and EBVincreased patients one year after transplant (Figs 2 and S2), as well as the incidence of opportunistic infections. Interestingly, we observed an increased incidence of acute rejection among EBVincreased (OR: 2.70 [1.11–6.10]; P = 0.019). Furthermore, we analysed whether a particular order of events in the association of acute rejection with EBV can be determined. Of 15 EBV+ patients with acute rejection, 10 (66.7%) patients demonstrated viraemia after the first acute rejection episode. Three patients (20.0%) had both adverse events within a time span of 2 weeks.

No significant effect of EBV was observed on eGFR one year post-transplant, nor on the incidence of loss to follow-up. EBV+ patients had a significantly higher incidence of CMV reactivation, while no clear effect association between EBV and BKV reactivation could be detected [4]. We did not observe a significant increase of fungal or bacterial opportunistic infections among EBV+ patients nor EBVincreased (Figs 2 and S2). Regarding the occurrence of anaemia, we observed within the EBV+ sub-cohort a significant negative correlation (ρ = −0.29; P = 0.009) between EBV viral load and haemoglobin levels one year after transplantation (Fig. 3).

*Patients with prolonged EBV reactivation had a higher incidence of BKV reactivation*

We investigated whether the 12 patients with EBVchron had distinctive transplant outcomes or complications, compared with the rest of the EBV+ sub-group. There was no evidence of alterations of the graft function; no difference was observed in the incidence of acute rejection, CMV, bacterial and fungal infections either (data not shown). However, there was an increase in BKV reactivation incidence (OR: 4.69 [0.92–46.67]; P = 0.059), when comparing EBVchron with the rest of the EBV+ sub-group.

**Discussion**

We have performed a characterization of the risk factors and associated complications of EBV reactivation in the context of renal transplantation. Despite relatively frequent reactivation of EBV during the first post-transplant year, no clear worsening of transplantation outcome was observed. Only a significant association with increased incidence of acute rejection and a correlation with low haemoglobin values were observed. Furthermore, we identified pre-transplant EBV shedding, male sex and, tentatively, increased LAG3 and CD200 gene expression two weeks post-transplant as potential predictors of EBV reactivation.

One of our main findings is the importance of pre-transplant EBV load as a post-transplant predictor. Self-limiting, asymptomatic EBV shedding in saliva or blood is common phenomena in healthy individuals, with great degree of inter- and intra-individual variation [19–21]. Importantly, both renal insufficiency and haemodialysis have a systemic impact on the immune...
response, potentially leading to reactivation events of latent pathogens [22,23]. Because of this, a substantial incidence of EBV pre-transplant shedding was expected [10]. Verghese et al., to our knowledge the only study in this topic in the literature, did not observe any such association between pre-transplant shedding and post-transplant reactivation. On the other hand, in our study the patients with pre-transplant EBV shedding were prone to four times higher incidence of post-transplant reactivation [10]. The reason for the discrepancy could lie on the small sample analysed by Verghese et al. [10].

Of note, our observed values for pre-transplant EBV incidence are remarkably similar to those by Verghese et al. Thus, they found a prevalence of 5%, compared with 6% in our study. The observed post-transplant incidence was also very similar – 21% vs. 18%. Regarding other studies on post-transplantation incidence, a wide range of EBV incidences (13–48%) has been observed after solid organ transplantation [3,19,24–26]. The large degree of inter-study variation can be due to differences in immunosuppressive regimes as well as the use of non-standardized quantitative PCR tests in several studies in the literature (including this work). The latter complicates the comparison between the results of the literature. However, it should be noted that our observed incidence for post-transplant EBV was in the lower range of the literature, so that an overestimation of EBV incidence seems unlikely [3,19,24–26].

We also identified potential associations of EBV reactivation with clinical alterations. We detected a significant correlation of EBV viral load with reduced haemoglobin levels. Furthermore, a significantly increased incidence of acute rejection was found. This is in line with our previous report demonstrating an association between EBV reactivation and transplant rejection [27]. With respect to their timeline, our data suggest that acute rejection precedes EBV reactivation in most cases. Anti-rejection therapy might play an important role in diminishing immune surveillance and accelerating disruption of EBV latency in patients with acute rejection.

Our data showed a tendency towards increased expression of the genes LAG3 and CD200 before EBV viraemia. These genes play a role in the regulation of the immune response and have a co-inhibitory effect on T-cell-mediated immunity [28,29]. A potential association with opportunistic infections such as EBV would

Figure 3 Correlation of peak viral among EBV⁺ patients with haemoglobin values one year post-transplant. Haemoglobin values below 14 g/dl for males and below 12 g/dl for females are considered indicative for anaemia.
therefore be not surprising. In fact, CD200 is differentially expressed in EBV-associated PTLD, while LAG3 is highly expressed in EBV+ gastric cancers [30,31]. However, it should be noted that no significant association of the genes with EBV viraemia was observed when adjusting for multiple testing. Therefore, further studies are paramount to confirm this hypothetical association.

Regarding sex effects, male patients suffered from a significantly higher incidence of EBV reactivation in our cohort. This is in disagreement with previous epidemiological studies with smaller cohorts [32–34].

There are limitations in our study. First, no correction for multiple testing was performed outside of the marker panel analysis, with the goal of capturing the maximum number of potential associations [35–37]. Therefore, the results we present here should be reproduced in confirmatory studies to consider them definitive. Secondly, this study was performed on a low immunological risk cohort of a randomized controlled trial [11]. Therefore, it cannot be considered to be representative for the general transplant recipient population. Thirdly, as explained before, viral load was measured employing an in-house protocol, instead of the WHO standard. This makes the comparison with the viral load in other studies difficult. Fourthly, the study population, because of their low immunological risk, was not at high risk for EBV-related complications. Accordingly, we observed relatively low replication values, despite the high incidence of reactivation. This limits the relevance of the conclusions on clinically relevant complications associated with EBV reactivation. Lastly, the follow-up time of the study does not allow for an evaluation of the incidence of PTLD, since most cases occur after the first post-transplantation year [38].

In spite of the aforementioned limitations, we have performed a comprehensive analysis of the risk factors and clinical complications of EBV reactivation in a large multi-centre cohort. Our analysis suggests pretransplant EBV shedding as an important risk factor associated with post-transplant EBV reactivation. Because of this, we hypothesize that EBV shedding could be employed as a biomarker for personalized immunosuppressive therapy. Furthermore, despite the association with decreased haemoglobin levels, EBV reactivations do not severely influence the post-transplant outcome in the short-term follow-up. An association between EBV and acute rejection can be explained by the diminished antiviral immune surveillance during anti-rejection immunosuppressive therapy. We consider that our results support the employed immunosuppressive regimes as relatively safe regarding EBV-associated complications. However, since

| Variable                              | Variable type | Cohort (N = 512) |
|---------------------------------------|---------------|------------------|
| Male sex                              | Categorical   | 327 (63.9%)      |
| Age at pre-Tx (years)                 | Continuous    | 56 [46–64]      |
| Weight at pre-Tx (kg)                 | Continuous    | 77.2 [68.1–88.6]|
| BMI at pre-Tx (kg/m²)                 | Continuous    | 25.8 [23.2–29.0]|
| Patient with previous transplant      | Categorical   | 490 (95.7%)      |
| Living donor                          | Categorical   | 65 (12.7%)       |
| Age of donor (years)                  | Continuous    | 55 [46–65]      |
| Expanded criteria donor*              | Categorical   | 221 (43.2%)      |
| Total HLA mismatches                  | Continuous    | 3 [2–4]         |
| Cold ischaemia time (min)             | Continuous    | 619 [416–840]   |
| Immunosuppression                     |               |                 |
| basiliximab + MMF + tacrolimus + corticosteroids | Categorical | 185 (36.1%)    |
| basiliximab + MMF + tacrolimus        | Categorical   | 166 (32.4%)      |
| rATG + MMF + tacrolimus               | Categorical   | 161 (31.4%)      |
| VGCV Prophylaxis                      | Categorical   | 299 (58.4%)      |
| Acute rejection                       | Categorical   | 56 (10.9%)       |
| Severe BKV viraemia (>10,000 copies/ml)| Categorical | 59 (11.5%)      |
| Severe CMV viraemia (>10,000 copies/ml)| Categorical | 18 (3.5%)       |
| eGFR-2w (ml/min-1.73 m²)              | Continuous    | 32.1 [18.1–46.2]|
| eGFR one year post-transplant (ml/min-1.73 m²) | Continuous | 47.6 [35.0–60.8]|

*The expanded criteria comprise a donor age greater than 60 years, or an age greater than 50 years combined with at least two of the following factors: cerebrovascular accident as the cause of death, hypertension, or a serum creatinine level of more than 1.5 mg/dl [11].
the incidence of PTLD increases with the transplant age, further follow-up studies are required to explore EBV pathogenicity in the long-term post-transplant course.

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Conflict of interest
The authors have no conflicts of interest to declare.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Results of the prediction of EBV employing the marker subsets. Each row represents a marker subset, each column represents the EBV group employed for comparison (either EBV+ or EBVincreased) and the visit of reference. The numbers and colour coding indicate the quality of achieved prediction, as estimated employing the BACC. NA values correspond to marker subsets for which not enough values were available for the EBV group to attempt prediction.

Figure S2. Forest plot comparing EBVincreased versus EBV− patients for selected demographic factors, outcomes and transplant complications. The square denotes the odds ratio, whereas an odds ratio over 1 means an increased higher incidence among EBVincreased patients; the grey lines denote the 95% confidence interval.

Figure S3. Expression of the co-inhibitory genes LAG3 and CD200 in the patient sub-cohorts two weeks post-transplant. The left column shows the comparison between EBV− and EBV+, while the right column shows the comparison between EBV− and EBVincreased. Note that the shown P values are not adjusted for multiple testing, for the adjusted P values see Tables S1 and S2.

Table S1. Results for the association of all measured markers with EBV+. The values of each measured marker for the EBV+ and the EBV− patients are shown, as well as the P value of the difference and the adjusted P value for each sub-cohort.

Table S2. Results for the association of all measured markers with EBVincreased. The values of each measured marker for the EBVincreased and the EBV− patients are shown, as well as the P value of the difference and adjusted P value for each sub-cohort.

Data S1. Detailed description of the methods employed for measuring the bio-marker panel and the statistical methods.

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