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ORIGINAL ARTICLE

Clinical implications of quantitative real time-polymerase chain reaction of parvovirus B19 in kidney transplant recipients – a prospective study

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Summary

This prospective study was designed to investigate the clinically significant level of parvovirus B19 viral load using quantitative real-time (RT) polymerase-chain reaction (PCR) in kidney transplantation (KT) recipients. One hundred forty-three adult recipients who underwent their first KT between November 2003 and October 2005 were enrolled. Six blood samples (the first taken preoperatively, subsequent samples taken every 4 weeks for 20 weeks) were taken from each patient for parvovirus B19 DNA RT-PCR analysis. All recipients were diligently followed for 1 year post-transplant. One hundred sixty-eight of the 715 (23.5%) postoperative samples were positive for parvovirus B19 PCR. Eighty-four of the 143 KT recipients (58.7%) showed at least one positive PCR. Sixteen of the 143 (11.1%) KT recipients had sustained severe anemia (SSA) with hemoglobin lower than 7.0 g/dl, after 4 weeks post-transplant. The incidence of SSA in recipients with a titer higher than 1×10^6 copies/5 μl whole blood was significantly higher than those with a negative or low titer ($P < 0.001$, positive predictive value 84.6%, negative predictive value 96.2%). In conclusion, a high titer of parvovirus B19 DNA higher than 1×10^6 copies/5 μl whole blood in KT recipients was related with SSA after 4 weeks post-transplant.

Introduction

Human parvovirus B19 is a small, non enveloped single-stranded DNA virus. Parvovirus B19 infection in organ transplant recipients may result from either the reactivation of latent infection, or can be acquired via primary infection subsequent to nosocomial spread, parenteral transmission by blood cell transfusions, infusion of contaminated products or by donor transmission via the transplanted organ [1]. The incidence of parvovirus B19 infection in kidney transplantation (KT) recipients has been reported to range between 1.8–31.1% [2–4], and in recipients presenting with anemia as high as 23–44% [5,6]. Parvovirus B19 infection have been reported in patients presenting with anemia, pure red cell aplasia (PRCA), pancytopenia [7–11] and has also been implicated in renal allograft dysfunction in KT recipients [12].

The diagnosis or monitoring of clinically significant parvovirus B19 infections has limitations in transplant recipients. In immunocompromised patients, serologic tests are unreliable and cannot be used to determine the activity and severity of parvovirus B19 infection. Recent reports suggest that direct detection of parvovirus B19 DNA by polymerase-chain reaction (PCR) is a sensitive

diagnostic tool for parvovirus B19 infection; however, the clinical implications of positive parvovirus B19 DNA detection by PCR remains unclear.

We performed this prospective study in order to evaluate the clinical significance of viral load of parvovirus B19 DNA measured by a quantitative real-time (RT) PCR technique in KT recipients with sustained anemia and to determine how viral titer loads can be used as a monitoring tool of this infection.

Patients and methods

Patients

Between November 2003 and October 2005, 143 consecutive adult recipients aged 15 years and above, undergoing their first KT from living or deceased donor at Samsung Medical Center, were enrolled in this prospective study. Multi-organ transplantation or second KT recipients were excluded. All the patients in the study group received triple immunosuppressive therapy; cyclosporine A (Cipol N capsule®, Chong Kun Dang, Seoul, Korea) or tacrolimus (Prograft®, Astellas, Ireland), mycophenolate mofetil (Cellcept®; Roche, Basle, Switzerland) and steroids. The study protocol was scrutinized and approved by the of Samsung Medical Center's Institutional Review Board. A detailed informed consent after proper counseling was obtained from all the patients. Baseline and demographic data, and all medications including dose were noted prospectively in the data base at the study entry point. Postoperatively all the KT recipients in the study group were managed as per the center's routine protocol.

Study design

Each KT recipient in the study group had six blood samples taken for parvovirus B19 DNA RT-PCR; one sample was taken preoperatively, and the other samples were taken every 4 weeks for 20 weeks post-transplantation. All recipients were followed up for the first 1 year post-transplantation. During this period, laboratory profiles including serum creatinine and hemoglobin (Hb) levels were checked and significant clinical episodes such as allograft rejection were monitored and treated as per our routine protocol.

We defined anemia as Hb lower than 10.0 g/dl and the recipients with anemia were closely monitored and evaluated for the exclusion of hematologic disturbance and bleeding causes such as a hematuria or gastrointestinal bleeding. We defined sustained severe anemia (SSA) as Hb lower than 7.0 g/dl after 4 weeks post-transplantation in the absence of hematologic disturbances or bleeding focus such as hematuria or gastrointestinal bleeding. The primary endpoint was SSA between 4 weeks and 12 months post-transplantation.

The recipients with SSA and Hb lower than 5 g/dl refractory to conventional treatment (transfusion, iron or recombinant erythropoietin administration or immune suppression reduction) had a bone marrow biopsy performed and were subsequently given 400 mg/kg/day of intravenous immunoglobulin (IVIG, IV-Globulin S®; Green Cross Pharmacy, Yongin-si, Kyunggi-do, Korea) for 7 days.

Parvovirus B19 DNA quantification

Determination of parvovirus B19 DNA viral load was done by quantitative RT-PCR, using the LightCycler instrument with the LightCycler® B19 Quantification Kit (Roche Diagnostics, GmbH, Penzberg, Germany) according to the manufacturer's instructions. The kit provides parvovirus B19-specific primers and two hybridization probes labeled with fluorescent molecules. Hybridization leads to fluorescence resonance energy transfer between the two fluorophores, and the emitted light is measured by a LightCycler® instrument on channel F2/Back-F1. Real-time (during amplification) monitoring of fluorescence intensities, relative to external standards of known target concentrations, allows for quantification of the accumulating product. To monitor the efficiencies of the nucleic acid extraction and the PCR process, ICs are amplified with the same primers as the target but hybridized with probes carrying different fluorophores. The fluorescence emitted from the IC-specific probes is measured on channel F3/Back-F1. The raw data created by either qPCR test were analyzed with LightCycler® Software version 3.5 (Roche). Crossing points and calculated concentrations were obtained using the second derivative maximum method coupled with proportional baseline adjustment. This method calculates the fractional cycle number of the crossing-point value of each sample automatically and thus ensures that the method is independent of any user-related influences. A previously generated color compensation file was activated during the LightCycler® run to reduce flow-through signal from other channels.

Statistical analysis

Continuous variables were compared using either the independent *t*-test or Mann–Whitney test according to Shapiro–Wilk test. Nominal variables from the KT recipient groups were analyzed by either Pearson's chi-squared test or Fisher's exact test. The significant level for all tests was set at 5%. All the analyses were performed using SAS software 9.1 (SAS Institute Inc, Cary, NC, USA).

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Results

Incidence and clinical features of parvovirus B19 infection and severe anemia

Parvovirus B19 PCR was positive in 14/143 (9.8%) of the preoperative and 168/715 (23.5%) of the postoperative samples. Eighty-four of 143 (58.7%) KT recipients showed at least one positive parvovirus B19 DNA PCR, and 36 (25.2%) of them had more than one positive parvovirus B19 DNA PCR. At 4, 8, 12, 16 and 20 weeks post-transplantation, positive rates of parvovirus B19 PCR were 28%, 21.7%, 26.6%, 23.8% and 21.7% respectively (Fig. 1a). Figure 1b shows the titers of parvovirus B19 DNA RT-PCR in KT recipients with parvovirus B19 infection according to time after transplantation.

Seventeen of the 143 (11.9%) patients and 12/143 (8.4%) patients had anemia at 6 months and 12 months post-transplant respectively. Between 4 weeks and 12 months post-transplant, 16/143 (11.1%) had SSA (Hb < 7.0 g/dl). The median interval between KT and the occurrence of the lowest Hb in the recipients with SSA was 47 days post-transplantation (interquartile range 34.25–112.25). The median of the highest parvovirus B19 DNA titers in recipients with one or more positive PCR were 36.1 copies/5 μl whole blood (interquartile range $21.3 \sim 1.4 \times 10^2$) ($n = 71$) in no-severe-anemia group and 3.9×10^7 copies/5 μl whole blood (interquartile range $1.0 \times 10^6 \sim 1.1 \times 10^9$) ($n = 13$) in sustained severe anemia group. Figure 2 shows median levels of hemoglobin and parvovirus B19 DNA PCR titers of the recipients with or without SSA.

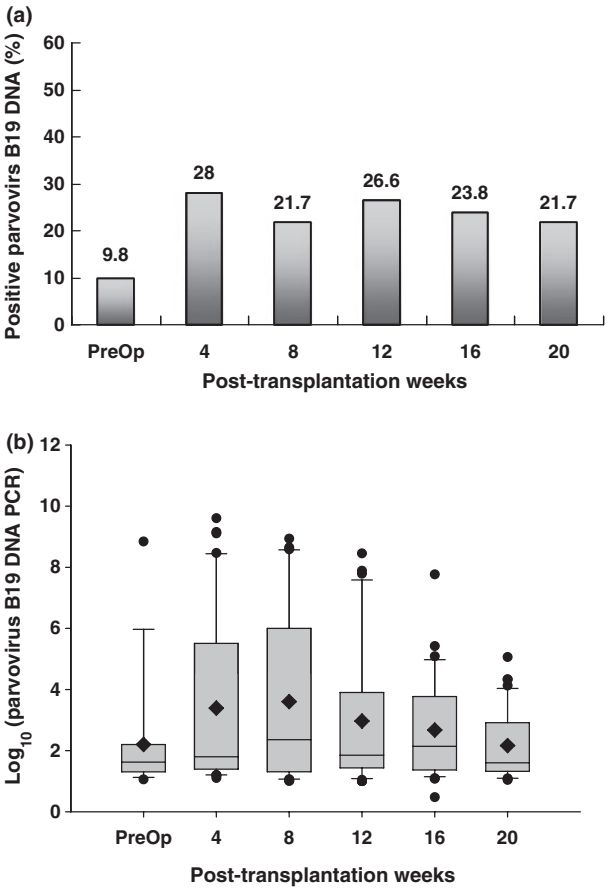

Figure 1 Quantitative parvovirus B19 DNA RT-PCR during the first 20 weeks post-transplantation. (a) Positive rate of the KT recipients. (b) Titers of parvovirus B19 DNA RT-PCR in KT recipients with parvovirus B19 infection. The distributions of viral titer in the KT recipients with positive parvovirus B19 PCR are shown as box plots with means of titer (♦) according to post-transplantation weeks.

Clinical course of the recipients with sustained severe anemia

In this study, 16 recipients presented with SSA (Fig. 3). The median level of serum iron of the recipients with SSA was 68.5 μg/dl (interquartile range 45.3 ∼ 107.3; reference range 50 ∼ 170). The median level of and total iron-binding capacity was 258 μg/dl (interquartile range 244 ∼ 318; reference range 250 ∼ 425). The levels of serum ferritin, folate and vitamin B12 were within the normal range or above lower limit in all the 16 recipients.

Five had negative or low titer in parvovirus B19 PCR detection. They were cautiously followed up under the monitoring of parvovirus B19 PCR titers and recovered spontaneously. Eleven had high titer of parvovirus B19 PCR and received conventional treatment including iron, recombinant erythropoietin administration, or transfusion. After the conservative management, the parvovirus PCR titers decreased and converted to negative and five recipients recovered from SSA.

In the 11 recipients with SSA and high titer, six recipients had extremely severe anemia with Hb lower than 5 g/dl and were refractory to treatment including immune suppression reduction. They underwent bone marrow biopsy. In three recipients bone marrow biopsy showed isolated erythroid hypoplasia and giant pronormoblasts with intranuclear inclusions, which is suggestive of PRCA resulting from parvovirus B19 infection. Their parvovirus B19 titers at diagnosis of PRCA were 867 200 000, 1 422 000 000 and 15 500 000 copies/5 μl whole blood. In the other three patients, the bone marrow was consistent with erythroid hypoplasia. All of these patients were treated with intravenous immunoglobulin (IVIG, 400 mg/kg/day) for 7 days. All the recipients responded to IVIG treatment; parvovirus B19 DNA titer decreased with the rise of Hb to a higher value than 10 g/dl with time. Figure 4 shows the profiles of Hb and parvovirus B19 DNA

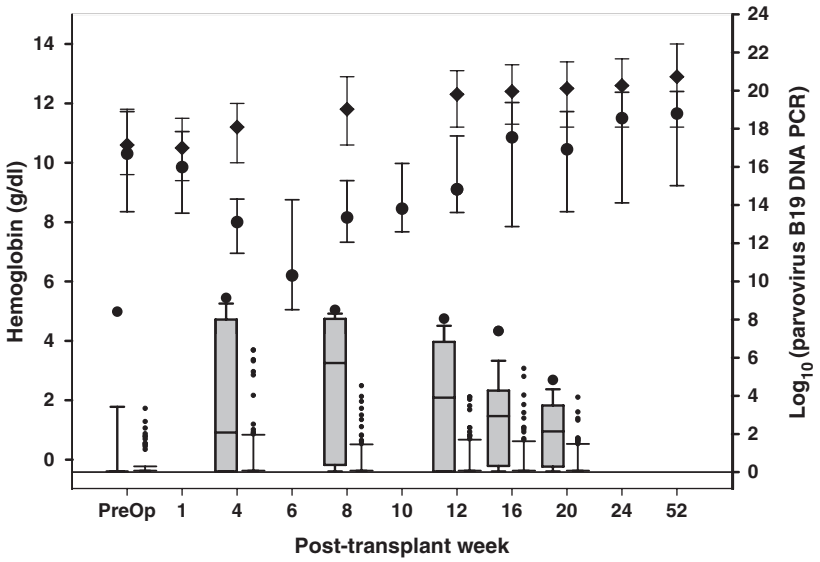

Figure 2 Hemoglobin level and parvovirus B19 DNA PCR titer of the recipients according to the presence of sustained severe anemia. Upper part of Fig. 3 shows median levels of hemoglobin with interquartile range (25 percentile, 75 percentile). Diamond (◆) means a median hemoglobin level of the recipients without sustained severe anemia (n = 127) and circle (●) does a median hemoglobin level of the recipients with sustained severe anemia (n = 16). Lower part of the figure shows Log_{10} (parvovirus B19 DNA PCR titer) with boxplots. The left boxplots at pretransplant and post-transplant 4, 8, 12, 16 and 20 weeks present the distribution of parvovirus B19 DNA PCR titers in the recipients with sustained severe anemia (n = 16) and the right boxplots do the distribution in the recipients without sustained severe anemia (n = 127).

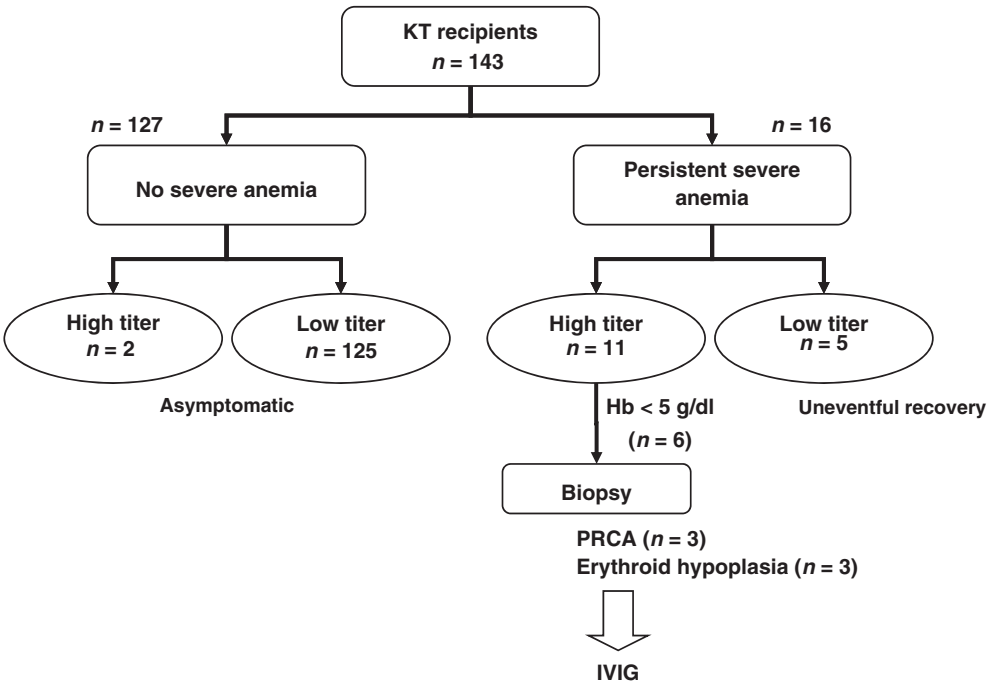

Figure 3 Clinical manifestation of persistent severe anemia in kidney transplantation (KT) recipients. High Titer = titer more than 1×10^6 copies/5 μl whole blood by parvovirus B19 DNA RT-PCR, Low Titer = titer less than 1×10^6 copies/5 μl whole blood by parvovirus B19 DNA RT-PCR (including negative in PCR), PRCA = pure red cell aplasia.

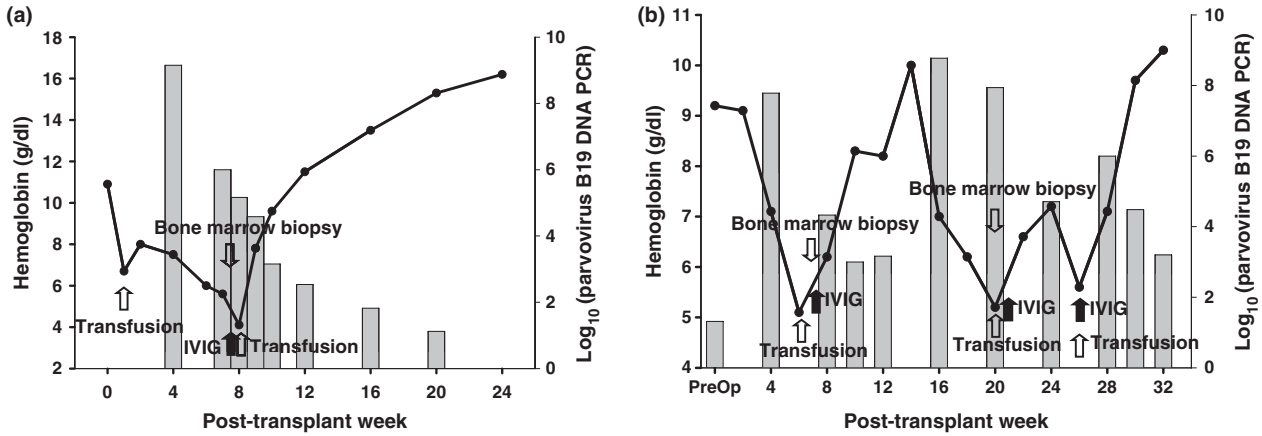

Figure 4 Profiles of hemoglobin and parvovirus B19 DNA titers of two recipients (panels a and b) with pure red cell aplasia. Line with dots shows the level of hemoglobin (g/dl) and bars do Log_{10}(Parvovirus B19 DNA PCR titer).

titers of two of the recipients, who were diagnosed as PRCA. One patient (Fig. 4b) presented with repeated severe anemia after IVIG treatment and was diagnosed as relapsing PRCA by bone marrow biopsy. This patient recovered after additional IVIG treatment at 7 months post-transplant.

Table 1. Demographic findings of the recipients according to the occurrence of sustained severe anemia.

	No severe anemia group (n = 127)	Sustained severe anemia† group (n = 16)	P-value
Age (year) (mean ± SD)	41.4 ± 10.2	39.8 ± 107	0.560‡
Gender (Male:Female)	73:54	5:11	0.047§
Donor (Living:Deceased) (case)	104:23	12:4	0.504¶
HLA mismatch (0–2:3–4) (case)	66:61	11:5	0.204§
Immunosuppression (case)			
Anti-thymoglobulin induction	14 (9.8%)	1 (15.8%)	1.000¶
Tacrolimus/Cyclosporine	37:90	7:9	0.257¶
Rejection episode (case)	32 (25.0%)	7 (43.8%)	0.139¶
Hemoglobin (g/dl) (mean ± SD)			
Pretransplant	10.7 ± 1.6	10.0 ± 2.0	0.141‡
At post-transplant 6 months	12.5 ± 1.6	10.7 ± 2.6	<0.001‡
At post-transplant 12 months	12.8 ± 1.9	11.0 ± 2.4	0.001‡
Anemia* (case)			
At post-transplant 6 months	11 (8.7%)	6 (37.6%)	0.004¶
At post-transplant 12 months	8 (6.3%)	4 (25.0%)	0.030¶
Parvovirus B19 titer (case)			
Pretransplant positivity	12 (9.4%)	2 (12.5%)	0.658¶
Positivity	71 (55.9%)	13 (81.3%)	0.052§
High Titer > 10^6 copies/5 μl	2 (1.6%)	11 (68.8%)	<0.001¶
Multiple High Titer	0 (0%)	7 (43.8%)	<0.001¶

*Anemia was defined as hemoglobin lower than 10.0 g/dl.
†Sustained severe anemia was defined as hemoglobin lower than 7.0 g/dl after post-transplantation 4 weeks.
‡Independent samples t-test.
§Pearson's chi-squared test.
¶Fisher's exact test.

Feasibility of parvovirus B19 DNA quantification by RT-PCR in PSA

Analysis for parvovirus B19 DNA quantification in sustained severe anemia

We investigated the maximum value of parvovirus B19 DNA quantification using responses at 4, 8, 12, 16, and 20 weeks post-transplantation. To assess the difference between 16 patients with SSA and 127 patients without SSA, we used a two-part model for data wherein a clump of 0 observations was used. This model is composed of a 2×2 contingency table and Wilcoxon's two-sample test [13,14]. The test indicated that the quantity of parvovirus B19 DNA in the recipients with SSA was significantly different from that in the recipients without SSA ($\chi^2 = 27.47$ with 2 d.f. and P-value = 0.0001).

Cut-off value of quantitative parvovirus B19 DNA RT-PCR

For quantitative parvovirus B19 DNA RT-PCR, the cut-off value for the probability of SSA occurring was calculated from the data of 143 recipients. This value was obtained using the minimum P-value approach with Fisher's exact test and the Chi-squared test. The cut-off value was 1×10^6 DNA copies/5 μl whole blood and was corrected by the Miller and Siegmund method ($P < 0.0001$) [15].

Clinical significance of parvovirus B19 DNA quantification and SSA

One hundred forty-three recipients were divided into two groups based on the occurrence of SSA with Hb lower

Table 2. The clinical significance according to parvovirus B19 DNA PCR quantification.

Parvovirus B19 DNA PCR	Anemia* at post-transplant 6 months			Anemia at post-transplant 12 months			Sustained severe anemia†		
	Yes	No	*P*-value‡	Yes	No	*P*-value‡	Yes	No	*P*-value
Positivity									
Positive	13	71	0.228**	9	75	0.720††	13	71	0.052**
Negative	4	55		3	56		3	56	
Titer§									
High titer	3	10	0.374††	1	12	1.000††	11	2	<0.001††
Low titer	14	116		11	119		5	125	
Mutiplicity¶									
Multiple high titer	1	6	1.000††	1	6	0.932††	7	0	<0.001††
Single high or low titer	16	120		11	125		9	127	

*Anemia was defined as Hb lower than 10.0 g/dl.
†Sustained severe anemia was defined as Hb lower than 7.0 g/dl after post-transplantation 4 weeks within 12 months post-transplantation.
‡*P*-value was corrected by Bonferroni's method.
§All the recipients were divided according to the titer in parvovirus B19 DNA PCR as high titer group showing titer higher than 10^6 copies/5 μl whole blood at least one time and low titer group.
¶All the recipients were divided according to the number of titer higher than 10^6 copies/5 μl whole blood as multiple high titer group titer higher than 10^6 copies/5 μl whole blood more than one times and single high or low titer group.
**Pearson's chi-squared test.
††Fisher's exact test.

than 7.0 g/dl between 4 weeks and 12 months post-transplantation i.e. no-severe-anemia group ($n = 127$) vs. SSA group ($n = 16$) and the clinical significance of parvovirus B19 DNA viral load determined by quantitative RT-PCR was evaluated (Table 1). Age, donor source, immune suppression, pretransplant Hb, pretransplant parvovirus B19 DNA positive detection and rejection episodes with steroid pulse therapy showed no significant statistical difference between the two groups ($P > 0.05$).

Table 2 shows the analysis of the clinical significance according to parvovirus B19 DNA PCR quantification. The positive rate of parvovirus B19 DNA (one or more positive samples) was higher in the SSA group, but was not statistically significant ($P = 0.052$). However, high-titer detection of parvovirus B19 DNA greater than 1×10^6 DNA copies/5 μl whole blood (estimated cut-off value) was significantly different between the two groups ($P < 0.001$). All the recipients who had multiple high-titer detection presented with SSA. Single determination of quantitative PCR above the cut-off value showed a positive predictive value of 84.6% (11/13) and a negative predictive value of 96.2% (125/130) for the occurrence of SSA within 1 year post-transplant. Positivity of parvovirus B19 PCR, or single or multiple high titer of parvovirus B19 DNA PCR was not associated with the presence of anemia at post-transplant 6 or 12 months.

Discussion

Anemia in KT recipients is a common complication subsequent to acute or chronic blood loss, decreased erythropoiesis because of immunosuppressive agent, infection or decreased erythropoietin production from renal dysfunction [16,17].

In this study, the positive rates among all postoperative blood samples for parvovirus B19 PCR (23.5%) and the detection rate (58.7%) of parvovirus B19 infection with at least one positive PCR during the first 20 weeks post-transplantation were much higher than those reported in previous studies (1.8–44%) [2–5]. PRCA was detected in three out of the six KT recipients who underwent bone marrow examination. This is a considerably high incidence because to date, less than 50 cases of parvovirus B19-related aplastic anemia in KT recipients have been reported [6].

A higher incidence of parvovirus B19 infection was noted in this study as compared with the previous reports (including the one from this center); the reasons for this are not clear. Multiple sampling and higher sensitivity of quantitative RT-PCR as compared with qualitative method may be some of the causes [18]. The relatively higher preoperative positive rate in PCR (9.8%) also to some extent explains the higher incidence of post-transplant parvovirus B19 infection. Anemia is one of the

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common complications in KT, and the monitoring for parvovirus B19 infection has not been introduced into routine protocol; parvovirus B19 infection may accordingly be underestimated and under-reported [4,19,20].

The clinical course of parvovirus B19 infections in the immunocompetent hosts is usually benign and self-limiting, and it is eliminated by the host's ability to generate specific antibodies. In contrast, in the immunocompromised hosts, this infection has been documented to be the cause of severe anemia and PRCA [7,8,10,11]. There have been several clinical suggestions for the diagnosis of parvovirus B19 infection and the treatment of parvovirus B19-related anemia in KT recipients [21–23]. However, there are few studies that have suggested relevant clinical strategies for the screening and monitoring of parvovirus B19 infection in KT recipients.

We previously reported on the clinical significance of parvovirus B19 infection by qualitative PCR in KT recipients [4]. Parvovirus B19 infection diagnosed positive in qualitative PCR can range in its clinical presentation from an asymptomatic infection to PRCA. KT recipients who had a positive PCR more than two consecutive times had significantly lower Hb levels than those with all negative or one positive PCR ($P < 0.0001$). This had very meager clinical implications and could not serve as a monitoring tool for the prediction of disease activity or severity or the determination of the treatment (positive predictive value 15.5%).

In this study, among the 130 KT recipients with negative or a low titer of PCR, only five (3.8%) had PSA, and they recovered spontaneously. Therefore, the recipients with a positive PCR but less than 1×10^6 copies/5 μl whole blood and moderate anemia might not require the intervention, but can be followed-up with conservative management by the monitoring of parvovirus B19.

In contrast, among the 13 recipients with quantitative PCR above the cut-off (1×10^6 copies/5 μl whole blood) at least once, 11 had SSA (Fig. 3). Three of these patients were confirmed by bone marrow biopsy as having PRCA. In the recipients who had multiple high titers and refractory severe anemia, bone marrow biopsy could be considered.

In this series, after 4 weeks post-transplantation, SSA occurred at a median interval of 47 days and the peaks viral titers were seen 4–12 weeks post-transplantation, after which they markedly dropped. Cavallo *et al.* reported that parvovirus B19 infection mainly occurred within 2.5 months post-transplantation, when immunosuppression is profound [5]. Parvovirus B19 infection does not seem to affect the prevalence of anemia in the late post-transplantation period; there was no statistical difference in Hb levels at 6 and 12 months post-transplantation (Table 2).

This study had limitations in the evaluation of the time-line event and the response of IVIG treatment because we had checked viral loads every 4 weeks post-transplantation. In most patients, SSA followed high-titer detection of parvovirus B19 DNA PCR. When some PRCA patients checked up parvovirus B19 DNA PCR titer more frequently than scheduled, after 7-day dose of IVIG, the titers of parvovirus B19 DNA PCR decreased over 2 or 4 weeks less than 1.0×10^6 copies/5 μl whole blood, and the level of hemoglobin had been already higher than 8 or 10 g/dl (Fig. 4). Follow-up parvovirus B19 DNA PCR could be helpful in the determination of the response to the therapy. Further evaluation with frequent check-up of parvovirus B19 DNA PCR should be performed.

The role of parvovirus B19 infections in influencing renal function remains controversial. It has been reported that specific glycosphingolipid receptors in kidney tissue play a role in direct and localized infection [24,25]. Parvovirus B19 infections have been reported to be associated with various glomerulopathies [12,26] or allograft rejection [3]. However, in this study, there was no parvovirus B19 related allograft dysfunction. Further studies to assess allograft dysfunction caused by parvovirus B19 infection with quantitative RT-PCR monitoring need to be carried out with long-term follow-up.

In conclusion, systematic monitoring of quantitative RT parvovirus B19 DNA PCR, showed parvovirus B19 infection to be more common than previously reported. In KT recipients, a parvovirus B19 DNA titer above 1×10^6 copies/5 μl whole blood in the early postoperative period revealed to be related with SSA after 4 weeks post-transplantation.

Authorship

Study concept and design: KSJ, JJW, LSK. Data collection: PJB, KDJ, CGS, CJM, JGO, Data analysis: PJB, WSY, Writing the manuscript: PJB, KDJ, Critical revision of the manuscript for important intellectual content: KCHD, KSJ, JJW, LSK.

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