Qualitative Detection of Human Cytomegalovirus DNA in the Plasma of Bone Marrow Transplant Recipients: Value as a Predictor of Disease Progression

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Objective: **The aim of this prospective study was to determine whether human cytomegalovirus (HCMV)-DNA detected by polymerase chain reaction (PCR) analysis in the plasma of bone marrow transplant (BMT) patients is a predictor of HCMV disease progression.**

Methods: **Plasma samples were collected from 15 patients who received allogenic BMTs. Each individual was sampled 1 week before and then weekly for 17 weeks after transplantation. The 270 plasma specimens were processed with a PCR method for detecting HCMV-DNA. Patients were also physically examined for signs or symptoms of HCMV-related disease.**

Results: **Eight (53.5%) of the 15 patients tested positive for HCMV-DNA. Two (25%) of these 8 individuals also had positive PCR findings before transplantation. Six (75%) of the 8 HCMV-DNA-positive patients had positive plasma-PCR results a week before clinical symptoms developed. The other 2 (25%) remained asymptomatic throughout their hospital stay. All 6 symptomatic cases were treated with ganciclovir, and 4 converted to negative plasma-PCR status at a median of 21 days. There was a significant correlation between PCR-detection of HCMV-DNA in plasma and presence of HCMV-related symptoms ($P < 0.01$).**

Conclusion: **Qualitative plasma-PCR analysis before and after bone marrow transplantation is a valuable way to screen for HCMV infection in BMT patients. Plasma-PCR monitoring of HCMV activity in this patient group might make it possible to administer an antiviral drug and thus reduce mortality. However, quantitative PCR is still considered the best way to accurately identify active HCMV infection and monitor treatment.**

Keywords: *Qualitative, Human cytomegalovirus, Bone marrow transplant patients, Plasma*

Bone marrow transplantation is now the method of choice for treating certain malignant and benign hematologic diseases, including thalassemia major [1,2]. However, infection of allografts with agents such as human cytomegalovirus (HCMV) is a serious problem in this patient group [1,3]. Human cytomegalovirus infection is one of the most common complications of transplantation and typically occurs between engraftment and day 120 in bone marrow transplant (BMT) recipients [4]. A study conducted in 1997 [5] indicated that approximately 60% of adults in developed countries and 100% of adults in developing countries are infected with this virus. Research done in Iran in 1994 showed that 89.7% of children under 14 years and 98.7% of adults were infected with HCMV [6].

Reactivation of latent HCMV infection in BMT recipients infected before transplantation or in transplanted tissue increases the risk of posttransplantation morbidity and mortality. Preemptive or early therapy is a promising approach for combating HCMV infection and may reduce the rates of morbidity and mortality in BMT recipients [7]. However, preemptive treatment for this virus requires a laboratory marker. A rapid and sensitive method for early diagnosis of HCMV is needed to

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detect this infection and monitor treatment. Virus isolation is a time-consuming procedure with low sensitivity, and results are affected by various environmental factors. Serology is not reliable for diagnosing HCMV disease, and interpretation of the results depends largely on the patient's immunologic condition.

BMT patients have a significantly lower mean HCMV viral load than solid-organ transplant patients [8]. Therefore, detection of HCMV-DNA in plasma may be a strong indication of active infection [9]. The ability to detect HCMV-DNA by polymerase chain reaction (PCR) analysis would help to significantly reduce HCMV disease in BMT transplant patients, although presence of HCMV-DNA in plasma is not specific for HCMV disease in BMT patients, whether or not it is useful as a predictor of progression. A positive result on conventional plasma-PCR indicates the presence of cell-free HCMV, which may indicate subsequent HCMV disease. It has been reported that PCR analysis is more sensitive than the pp65 test for detecting clinically significant HCMV infections that require ganciclovir treatment [10,11].

We conducted a cohort study to determine the prognostic value of PCR-detected HCMV-DNA in the plasma of BMT recipients.

Materials and Methods

Patients

Seventeen BMT recipients who received allogenic BMTs at Namazee Hospital, Shiraz University of Medical Sciences between May 2001 and September 2002 participated in the study. Two patients who died before day 21 after transplantation were excluded. The remaining patients were 10 males and 5 females aged 10-36 years. The indications for bone marrow transplantation included leukemia (n = 6), thalassemia major (n = 8), and aplastic anemia (n = 1). The HCMV serology status of each donor and recipient was determined prior to transplantation by enzyme immunoassay. Surveillance blood samples were collected 1 week before and then weekly for 17 weeks after transplantation. All recipients received filtered blood transfusions posttransplantation. Clinical data were recorded and analyzed prospectively by physicians who were blind to the laboratory data.

The patients with leukemia were prepared a conditioning regimen of busulfan 16 mg/kg and cyclophosphamide 200 mg/kg for 4 days before marrow transplantation. All 6 patients received methotrexate 15 mg/m^2 on the first day after transplantation and 10 mg/m^2 on days 3 and 6. These individuals also received folinic acid 5 mg IV every 6 hours on days 7 and 8 posttransplantation. To prevent acute graft-versus-host disease (GVHD), all 6 of these individuals received an immunosuppressive regimen of cyclosporine. This involved an IV dose of 5 mg/kg/day for 2 days before marrow transplantation for up to 6 days post transplantation, followed by 3 mg/kg/day IV or 12.5 mg/kg orally for up to 180 days posttransplantation.

For the 8 patients with thalassemia, the conditioning regimen was busulfan 16 mg/kg and cyclophosphamide 200 mg/kg for 4 days before marrow transplantation. As a modification of the conditioning regimen, antithymocyte globulin 10 mg/kg/day was also given to all these patients for 2 days before marrow transplantation and for up to 2 days posttransplantation. To prevent GVHD, these individuals were placed on an immunosuppression regimen of cyclosporine identical to the dosing schedule detailed above, except that the posttransplantation treatment lasted for up to 1 year.

The conditioning regimen for the aplastic anemia patient was cyclophosphamide 200 mg/kg for 4 days before marrow transplantation, and antithymocyte globulin 15 mg/kg/day 5 days before BMT transplantation and for up to 5 days posttransplantation.

As prophylaxis for HCMV and herpes virus infection, each BMT patient received acyclovir (15 mg/kg/day) 1 day before and for up to 180 days after transplantation. Sandoglobulin (500 mg/kg) IV was also given 1 day before, on days 8 and 21 posttransplantation, and then every 3 weeks for up to 6 months. Human cytomegalovirus-related disease was identified according to the criteria of Ljungman et al [12].

Specimens

A total of 270 plasma samples (one per week for 18 weeks) was collected from the 15 allogenic-BMT recipients. For each sampling, 10 mL of blood was collected into an EDTA tube by venipuncture, and the plasma was isolated by centrifugation. All the plasma samples were frozen at -20°C until they were analyzed.

DNA Extraction and PCR Amplification

One hundred microliters of each plasma sample was processed using a standard proteinase K and phenol-chloroform nucleic acid extraction method. The

samples from before and after transplantation were all analyzed by PCR. Amplification was performed using an HCMV-PCR detection kit (Cinna Gennic, Tehran, Iran). According to manufacturer's instructions, 5 μL of extracted DNA was added to 20 μL of a PCR mixture that contained primers that would amplify 222 bp in the region of the major immediate early gene. The PCR program was as follows: Samples were denatured for 2 minutes at 94ºC; then subjected to 44 cycles of 93ºC for 40 seconds, 62ºC for 40 seconds, and 72ºC for 40 seconds; and the final step was 72ºC for 10 minutes. Ten microliters of each amplified product was analyzed by conventional gel electrophoresis followed by ethidium bromide staining (10 μg/mL). Any plasma sample that was positive for HCMV-DNA was retested.

According to standard procedure for preventing contamination, each procedure for PCR processing was performed in physically separate rooms. Negative controls were used to ensure that carryover contamination did not occur.

Determination of the Sensitivity and Specificity of PCR

To assess the sensitivity of the PCR assay, template DNA was extracted from 10-fold serial dilutions of plasmid DNA (Cinna Gennic, Tehran, Iran. Originally supplied from Fermentas Life Sciences) that had been seeded into plasma samples that were negative for HCMV-DNA. Conventional gel electrophoresis and ethidium bromide staining were then used to analyze the PCR amplification products from these templates. The specificity of PCR was determined using DNA extracted from a herpes virus group other than HCMV and from uninfected Vero and Hep-II cell lines.

Statistical Analysis

All statistical testing was done using SPSS software version 9.0. Chi-square analysis and the two-tailed Fisher's exact test were used to analyze differences between results obtained by PCR method and presence or absence of HCMV-related symptoms.

Results

All but one of the 15 donors (93%) and all (100%) of the BMT recipients tested positive for HCMV antibodies.

Sensitivity and Specificity of PCR: The lowest limit at which PCR was able to detect HCMV-DNA in plasma was 10^2 copies of the selected 222-bp DNA segment per reaction tube. Regarding specificity for detecting HCMV-DNA, no amplification was noted on gel electrophoresis analysis.

Detection of HCMV-DNA in BMT Recipient Plasma: Polymerase chain reaction analysis of all the samples from before and after transplantation demonstrated HCMV-DNA in the plasma from 8 (53.5%) of the 15 patients. All of these individuals had thalassemia. Two (25%) of these 8 patients had posi-

Table 1. Clinical features and plasma-PCR findings in the 8 patients who were HCMV-DNA-positive at some point during the study.

Patient No.	Clinical symptoms	PCR pretransplantation	PCR posttransplantation	Indication for BM transplantation
1	F,G,S	(+)	(+)	Thalassemia major
2	N	(-)	(+)	Thalassemia major
3	N	(-)	(+)	Thalassemia major
4	G,S,E	(-)	(+)	Thalassemia major
5	F,G,S,U	(-)	(+)	Thalassemia major
6	F,P	(-)	(+)	Thalassemia major
7	F,G	(+)	(+)	Thalassemia major
8	F	(-)	(+)	Thalassemia major

Abbreviations: BM = bone marrow; E = retinitis; F = fever; G = gastrointestinal; HCMV = human cytomegalovirus; N = asymptomatic; P= pneumonia; PCR = polymerase chain reaction; S = skin lesion; U = urinary tract

Table 2. Results of plasma-PCR analysis for HCMV-DNA and HCMV-related symptoms in the 15 bone marrow transplant recipients who were studied.

PCR result	No. of patients	No. with HCMV-related symptoms	No. without symptoms
Positive	8/15 (53.3%)	6/8 (75%)	2/8 (25%)
Negative	7/15 (46.7%)	1/7 (14.2%)	6/7 (85.7%)
Total	15	7/15 (46.6%)	8/15(53.4%)

tive PCR findings before transplantation and remained HCMV-DNA-positive for up to 4 weeks after surgery. Ganciclovir treatment improved patients' condition. Concerning the remaining 6 (75%) HCMV-DNA-positive cases, 1 patient's plasma was positive for only 1 week, and the other 5 cases remained positive for 4 to 8 weeks after transplantation.

Six (75%) of the 8 patients with positive pPCR results exhibited symptoms of HCMV infection (Table 1). There was a significant correlation between PCR-detection of HCMV-DNA in plasma and presence of HCMV-related symptoms ($P < 0.01$). Interestingly, all 6 of the patients who developed clinical symptoms tested positive for HCMV-DNA 1 week before the symptoms appeared. The other 2 (25%) HCMV-DNA-positive patients remained asymptomatic throughout their time in the hospital. All 6 of the symptomatic infected patients were treated with ganciclovir, and 4 converted to negative pPCR status at a median of 21 days posttreatment.

Plasma-PCR testing detected no HCMV-DNA in 7 (46.7%) of the 15 BMT recipients investigated (Table 2). Two of these 7 patients showed no clinical signs or symptoms of any kind. One of the other 5 developed aspergillosis and one developed GVHD during the study. Two individuals developed both gastrointestinal signs during the first week posttransplantation, but these problems subsided 2 days after chemotherapy dosages were reduced. One patient developed HCMV-related symptoms including fever, diarrhea, and skin rash 6 weeks after transplantation, but these responded to ganciclovir treatment within 1 week.

Discussion

Patients with thalassemia major are predisposed to symptomatic HCMV infection. At present, qualitative PCR analysis of plasma is the method most widely used to achieve early detection of HCMV after transplantation [10]. In some situations, pPCR analysis is better than leukocyte PCR analysis for this purpose. This is especially true for leukopenic patients, who may have inadequate numbers of leukocytes for sensitive PCR detection of HCMV [10,11].

In this study, we used a simple and a rapid qualitative PCR assay for early detection of HCMV-DNA in plasma samples that were collected a week before and in the first 120 days after bone marrow transplantation. The results provide new insights that might help improve patient management.

The first finding of interest is that PCR analysis detected HCMV-DNA in the plasma of 2 of the 15 BMT patients in the days before transplantation. Both of these individuals had thalassemia major. However, previous research has suggested that this particular virus can be transmitted via blood transfusion from donors with latent infection [11]. Thalassemia patients require repeated transfusions, and such treatments may increase an individual's HCMV-DNA level. However, in more-recent studies, HCMV-DNA has been detected only in the blood of patients with active infection [11]. Our two patients who were positive prior to bone marrow transplantation developed HCMV disease 3 and 4 weeks posttransplantation, respectively. Ganciclovir treatment improved the clinical condition of only one of these two individuals. In the other case, the patient's clinical status improved without ganciclovir. This finding is consistent with results from studies of solid-organ transplant recipients [12,13].

To confirm that HCMV-DNA may be present in the peripheral blood of thalassemia patients, we PCR-tested plasma specimens from 20 individuals with this illness who were not in our study group (data not shown). The PCR findings revealed HCMV-DNA in 1 (5%) of these samples.

Our findings suggest that qualitative PCR detection of HCMV-DNA in the plasma of allogenic-BMT recipients before transplantation may be an important predictor of HCMV disease after transplantation. If this is true, then preemptive treatment of HCVM-DNA-positive BMT patients with ganciclovir may prevent HCMV disease. Quantitative PCR methods will help strengthen the correlation between pPCR findings for HCMV and symptomatic disease [12,14]. Also, more testing of preoperative clinical samples will be needed to clearly determine the risk of HCMV disease in patients who have positive pPCR results before transplantation.

In total, 8 of our 15 subjects had positive pPCR results for HCMV-DNA at some point during the study. As mentioned, two patients tested positive before transplantation. The other 6 cases (75% of all positives) had positive PCR findings at 4 to 5 weeks posttransplantation. This is the approximate period in which one would expect HCMV to become activated in a transplant recipient. During this period, numbers of $CD4^+$ and $CD8^+$ T cells drop significantly, and the immune system is unable to prevent HCMV activation [15,16]. Interestingly, pPCR detected HCMV-DNA in 6 of our 8 (75%) positive patients a week

before clinical signs and symptoms of HCMV disease appeared. This rate is 5% higher than that reported by Evans et al [16]. Two (25%) of our HCMV-DNA-positive patients remained asymptomatic throughout the entire study period, and previous reports have noted this as well [12,17,18]. Our two asymptomatic patients recovered (converted to negative HCMV-DNA status) without ganciclovir treatment. Two factors may explain positive PCR findings for this virus in asymptomatic patients. First, a highly sensitive PCR assay might detect a low copy number of amplified HCMV-DNA segments in an individual with latent HCMV infection. Second, gradual strengthening of the immune system in the weeks after transplantation may prevent HCMV activation.

In a recent study, investigators used real-time PCR to assess the advantages of detecting HCMV-DNA in plasma [9]. The PCR findings were compared with findings for pp65 antigenemia. The authors showed that HCMV-DNA is detected on pPCR in BMT recipients before such individuals test positive for pp65 antigen. Our results are in accordance with these findings, since pPCR was positive at least a week before the onset of HCMV disease in all the symptomatic cases. Antigenemia is usually detected at the same time symptoms of HCMV disease appear.

Plasma-PCR testing revealed no HCMV-DNA in 7 (46.5%) of the 15 BMT recipients in our study. Six weeks after transplantation, one of these 7 patients developed HCMV-related symptoms, and these responded to ganciclovir treatment. There are two possible explanations for the negative pPCR results in the face of clinical symptoms in this case: the patient might have had HCMV disease with a low plasma DNA level or might have been infected with another type of viral infection.

In conclusion, this study shows that qualitative pPCR analysis before and after bone marrow transplantation is a valuable way to screen for HCMV infection. Our findings suggest that PCR-detection of HCMV-DNA in the plasma of allogenic-BMT recipients before transplantation may be an important predictor of HCMV disease after transplantation. Plasma-PCR analysis for HCMV-DNA would be an effective way to decrease mortality in BMT patients, as it would permit early diagnosis of HCMV infection and enable physicians to use preemptive therapy or treat a confirmed infection with antiviral drugs. However, quantitative PCR is still considered the best way to accurately identify active HCMV infection and monitor treatment.

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