Quantitative Analysis of Epstein-Barr Virus DNA Load in Bone Marrow Transplant Recipients By Using Real-Time PCR

Sabri SH.,* **Ghaffari H., Sadeghizadeh M., Alimoghaddam K., Ghavamzadeh A.**
Hematology-Oncology and Bone Marrow Transplantation Research Center, Shariati Hospital, Tehran, Iran.
**Corresponding Author*

Abstract
Introduction: Quantification of Epstein - Barr virus (EBV) in peripheral blood mononuclear cells (PBMNC) of allogenic bone marrow transplant (BMT) recipients is important because EBV-associated posttransplant Lymphoproliferative disease (PTLD) can occur after transplantation due to immunosuppression therapy.
Methods: To this end we chose Real-Time PCR using TaqMan probe. For the standard curve, we cloned BALF5 gene of EBV into a plasmid vector. After purification of the EBV-clone and calculation of plasmid copy number, the standard curve was constructed by using serial dilution of the plasmid clone.
Results: We were able to detect from 2 to 10^7 copies per 2×10^5 PBMNC with wide linear range. The mean EBV DNA copy number was 103.7 copies per 2×10^5 PBMNC. In this study, No patient of 35 BMT recipients (275 PBMNC samples) developed PTLD during five months follow up post transplant. EBV copy numbers in 22 samples (3 patients) out of 35 BMT recipients were higher than cut off value with symptoms like fever and pulomonary noddes (9%). The virus load in one patient in the last sample obtained was 72400 copies. We detected low levels of EBV DNA in 20 BMT patients (57/1%).
Conclusion: Real-Time PCR is useful to measure virus load in PBMNC. Detection of EBV in PBMNC samples may be valuable predictive marker to prognosis PTLD. Further studies need to determine accurate viral cut off value for treatment patients at risk for PTLD.

Key words: Epstein - Barr virus (EBV), Real-Time PCR, posttransplant Lymphoproliferative disease (PTLD)

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Introduction
Epstein – Barr virus (EBV) is a lymphotropic herpesvirus that infects preferentially B cells and occasionally other cell types, especially epithelial cells.(1) After primary infection, EBV establishes an asymptomatic latency state. In immuno compromised patients, like allogenic bone marrow transplant (BMT) recipients receiving immunosuppression to prevent graft rejection or graft – versus – host - disease, reactivation of EBV infection may lead to posttransplant lymphoproliferative disorders (PTLD).(2) Early diagnosis of PTLD is still a prerequisite for successful treatment, despite advances in therapy.(3,4) Recent studies have demonstrated a relationship between the extent of EBV load in peripheral blood mononuclear cells (PBMC) and the risk of developing PTLD.(4,5) Although PTLD patients usually exhibit uncommonly high levels of EBV DNA, new evidence that an increased viral load alone might not be related to PTLD development has emerged (6). Thus, an accepted level of EBV load predective of PTLD development has not been established as yet.
Conventional PCR only allows semiquantitative results and time – consuming hybridization and blotting steps are necessary after amplification. (7) These disadvantages have now been over come by the real-Time TaqMan PCR technology using Roche Light Cycler detection system. The assay has a very large dynamic range of target molecule determination because the real-Time measurement of the PCR product enables us to quantify the amplified products in the log phase of the reaction.(8)

In this study the real-Time PCR method was applied to the measurement of EBV DNA in PBMC samples of Allogenic BMT recipients.

Methods

Patients and samples: 275 samples of 35 allogenic BMT recipients were enrolled in this study (12 with AML, 8 with CML, 8 with ALL, 4 with A. Anemia, 1 with Thalassemia, 1 with Hodgkin disease and 1 with MDS). These patients were 9 to 65 years old (mean age 27.1 years). 27 out of 35 patients were mails (79.4%) and 7 patients were femails (20.6%).

EDTA –Treated blood was taken from the patients and the PBMC were seperated with Ficoll-paque. For the PCR assay, the DNA was extracted from the PBMC fraction by boiling method and stored at -20° C until use. We cloned a part of EBV BALF5 gene into a pTZ57R/T vector (PTZ-BALF5). After purification of the EBV clone and calculation of plasmid copy number, the standard curve was constructed by using serial dilution of plasmid clone (PTZ-BALF5). EBV copy number of patients samples were calculated automatically with the use of this standard curve.

Real - Time quantitative PCR with a fluorogenic probe. The PCR primers for this assay were selected in BALF5 gene encoding DNA polymerase.[5] The upstream and downstream primer sequences were 5´-CGGAAGCCCTCTGGACTTC-3´ and 5-CCCTGTTTATCCGATGGAATG-3´ respectively. A fluorogenic probe 5´-TGTACACGCACGAGAAATGCGCC-3´ with a sequence located between the PCR primers was synthesized by MWG company. The PCR reaction was performed using 100 ng of DNA from PBMC was added to a PCR mixture containing 5 mM Mgcl2, 200 µM dNTP Mix, 0.2 µM each primer , 0.1 µM fluorogenic probe, and 1.25 U of Taq enzyme (Roche company). Real-Time fluorescence measurements were taken and threshold cycle (CT) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit.

Results

The standard curve was constructed with serial dilution (10 to 107 copies) of the control plasmid. To confirm the specificity of the primers and probe, an EBV negative lymphoma cell line, was tested by this method. No one was positive for EBV DNA. Also the negative control always was without any fluorescence signal.

We estimated EBV DNA copy number in patients PBMC samples. The mean number of EBV DNA genome, was 103.7 copies of EBV DNA by using real-time TaqMan PCR.

In this study, most patients received posttransplant immune suppression to prevent graft-versus-host-disease (GVHD), usully with cyclosporine. By the way, we observed the occurrence of GVHD in 16 BMT recipients, but only in one of these cases EBV DNA load was higher than cut off value. No correlation was detected between EBV copy number and GVHD grade.

Discussion

The aim of this working was to setup a real-Time TaqMan PCR for detection of EBV DNA load in BMT recipients. This work has been performed for the first time in Iran. Real-time PCR with a fluorogenic probe is a technique which enables us to quantify a large number of amplified products rapidly and accurately[8] using roche light cycler system. We could detect EBV DNA in PBMC samples. Kimura et al and Niesters et al had demonstrated that real-time system has been applicable to quantitation of EBV load in patients with symptomatic EBV infections.[9,10] On the other hand, this method eliminates the precautions that must be taken with amplified products to avoid contamination because the method is done in absolutely sealed wells. This is an important improvement over the conventional PCR assays, which have considerable risks of carryover contamination. With its rapidness, accuracy and ability to handle many samples, the real-Time PCR assay should replace the quantitative PCR methods.[9]

Lymphoproliferative disorders after bone marrow and organ transplantation and among patients infected with AIDS are believed to result from uncontrolled proliferation of EBV transformed B-lymphocytes in the setting of immune dysfunction.[11] The incidence of allogenic bone marrow transplantation – related PTLD is low but because PTLD mortality rate is 50%-80% , diagnosis of disorder is very important for treatment strategies of PTLD.[12] Given the gen-

erally poor prognosis of patients with PTLD, early detection is important to maximize the chances of a successful outcome. EBV load is regarded as a useful indicator of the changes in viral dynamics that precede the development of PTLD. This test can also detect the increased viral replication associated with acute EBV disease.[13]

In our study, from 275 samples of 35 BMT patients, EBV load in 22 samples out of 3 patients were higher than cut off value. The virus load in one patient in the last sample obtained posttransplant was increased to 72400 copies. Also we observed low levels of EBV DNA genomes in 20 BMT patients (57.1%), but no patient developed PTLD during five months posttransplant. It is believed that the time to PTLD diagnosis posttransplant can be highly variable, ranging form a few months to several years.[12] So if our patients population was greater or the time of patients follow up posttransplant was longer, maybe EBV infection would lead to occurrence of PTLD. Finally, EBV DNA is frequently found in allogenic bone marrow transplant recipients. Patients with low EBV copy numbers may stay without clinical symptoms but those with high levels of EBV load are probabely at high risk of PTLD.

By the way, EBV DNA is a surrogate tumor biomarker in a wide variety of EBV – related disorders. Also EBV DNA is important in using of chemotherapeutic regimens and monoclonal antibodies such as rituximab in the treatment for these tumors. It may be particularly helpful in lymphomas in which clonal markers evaluable by PCR are not available. These include NK cell lymphomas and Hodgkin lymphoma, whereby clonal rearrangement of the immunoglubolin and T-cell receptor genes could not be applied for detection of minimal residual disease. So real - Time PCR and quantitation of EBV DNA load can be valuable and helpful in these cases. Further studies need to determine accurate viral cut off value for treatment patients at risk for PTLD.

References

1. Thorley-Lawson DA. Epstein-Barr virus: exploiting the immune system. Nat Rev Immunol. 2001; 1: 75 -82.
2. Au WU, Pang A, et al. Quantification of circulating Epstein-Barr virus (EBV) DNA in the diagnosis and monitoring of natural killer cell and EBV-positive lymphomas in immunocompetent patients. Blood. 2004; 104: 243-249.
3. Heslop HE, Roony CM. Adoptive cellular immunotherapy for EBV lymphoproliferative diseases. Immunol Rev. 1997; 157: 217-222.
4. Jabs WJ, Henning H, et al. Normalized quantification by Real-Time PCR of Epstein-Barr virus load in patients at risk for posttransplant lymphoproliferative disorders. J Clin Microbiol. 2001; 39: 564-569.
5. Bai XG, Hosler BB, et al. Quantitative polymeraise chain reaction for human herpes virus diagnosis and measurement of Epstein-Barr virus burden in posttransplant lymphoproliferative disorder. Clin chem. 1997; 43: 1843 – 1849.
6. Lucas KG, Burton RL, et al. Semiquantitative Epstein-Barr virus (EBV) polymerase chain reaction for the determination of patients at risk for EBV-induced lymphoproliferative disease after stem cell transplantation. Blood. 1998; 91: 3654-3661.
7. Rowe DT, Reyes N, et al. Use of quantitative competitive PCR to measure Epstein-Barr virus genome load in the peripheral blood of pediatric transplant patients with lymphoproliferative disorders. J Clin Microbiol. 1997; 35: 1612-1615.
8. Heid CA, Stevens KJ, et al. Real-Time quantitative PCR. Genome Res. 1996; 6: 986-994.
9. Kimura H, Morita M, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol. 1999; 37: 132-136.
10. Niesters HG, Van Esser J, Fries E, et al Development of a Real-Time quantiative assay for detection of Epstein - Barr virus. J. Clin. Microbiol. 2000; 38: 712-715.
11. Curtis RE, Travis LB, et al. Risk of lymphoproliferative disorders after bone marrow transplantation: a Multi-institutional study. Blood. 1999; 94: 2208-2216.
12. Vikas, D. Post-transplant lymphoproliferative disease, 2005.
13. Allen U, Tellier R, et al. Utility of semiquantitative polymerase chain reaction for Epstein-Barr virus to measure virus load in pediatric organ transplant recipients with and without posttransplant lymphoproliferative disease. 2001; 33: 145-151.