·CLINICAL RESEARCH·

Infection Status and Clinical Significance of Epstein-Barr Virus in Pediatric Leukemia —— A Report of 35 Cases

LU Yuan[1], SUN Li-Rong[1], PANG Xiu-Ying[1], LU Zhen-Hua[2], SUI Ai-Hua[2], LI Xue-Rong[1], SONG Ai-Qin[1]

1. Department of Pediatrics, Affiliated Hospital, Medical College, Qingdao University, Qingdao, Shandong, 266003, P. R. China
2. Molecular Biology Lab, Affiliated Hospital, Medical College, Qingdao University, Qingdao, Shandong, 266003, P. R. China

Correspondence to: LU Yuan
Tel: 86-532-82911313
E-mail: luyuan6967@yahoo.com.cn

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[ABSTRACT] **BACKGROUND & OBJECTIVE:** Epstein-Barr virus (EBV) is associated with genesis of many human tumors. This study was to detect EBV infection in pediatric leukemia, and to explore its clinical significance. **METHODS:** EBV DNA in peripheral blood mononuclear cells in 35 pediatric leukemia patients, including 26 cases of acute lymphoblastic leukemia (ALL) (24 received initial treatment and 2 received retreatment), 8 cases of acute non-lymphocytic leukemia (ANLL) and 1 case of chronic lymphocytic leukemia (CLL), and in 14 healthy children was detected by fluorescent quantitative polymerase chain reaction (FQ-PCR). Its clinical significance was analyzed according to the clinical manifestations, prednisone sensitivity test, and complete remission (CR) rate after induction chemotherapy. **RESULTS:** EBV DNA was detected in 8 (22.86%) of the 35 pediatric leukemia patients. The positive rate of EBV DNA was 26.92% (7/26) in ALL with quantity of $(5.144 \pm 6.91) \times 10^5$ copies/ml, and 12.5% (1/8) in ANLL patients with quantity of 4.031×10^3 copies/ml. No EBV DNA was detected in CLL patients and healthy controls. The occurrence rates of peripheral leukocytosis and hepatosplenomegaly were significantly higher in the patients with EBV infection than in the patients without EBV infection (P <0.001). In ALL, the rate of no response to prednisone was significantly higher in the patients with EBV infection than in the patients without EBV infection (100% vs. 26.32%, P =0.001); CR rate after induction chemotherapy was significantly lower in the patients with EBV infection than in the patients without EBV infection (28.57% vs. 84.21%, P=0.003). In ANLL, the differences of CR rate and relapse rate were not significant between the patients with and without EBV infection (P= 0.5). **CONCLUSIONS:** Pediatric leukemia patients with EBV infection have higher incidence of peripheral leukocytosis and hepatosplenomegaly. ALL patients with EBV infection have poor prednisone response and low CR rate.

KEYWORDS: Epstein-Barr virus; Leukemia, children; Fluorescent quantitative polymerase chain reaction

Introduction

Epstein-barr virus (EBV), as a human herpesvirus, is not only associated with diseases such as nasopharyngeal cancer [1], infectious mononucleosis[2], Burkitts lymphoma [3], but also with neoplastic hematologic disorder. In order to explore the EBV infection status in pediatric leukemia patients and its clinical significance, fluorescence-quantitative polymerase chain reaction (FQ-PCR) was adopted to quantify EBV DNA in peripheral blood mononuclear cells (PBMNCs) of 35 pediatric leukemia patients and 14 healthy controls.

Materias and Methods

Patients and samples

EBV DNA in peripheral blood mononuclear cells of 35 pediatric leukemia patients, including 26 cases of acute lymphoblastic leukemia (ALL) (24 received initial treatment and 2 received re-treatment), 8 cases of acute non-lymphocytic leukemia (ANLL), 1 case of chronic lymphocytic leukemia (CLL) and 14 healthy controls, was detected by FQ-PCR. All patients were from the Department of Pediatrics of the Medical School Hospital of Qingdao University from Jan 2004 to Mar 2006. Twenty-four cases were newly diagnosed and 2 cases were relapsed; 14 patients were males, and 12 were females [mean age 6.5 (range 1 to 12) years]. According to immunologic classification, 16 were early pre B-ALL, and 8 were pre B-ALL, 1 was B-ALL, and 1 was My+-ALL. In ANLL, 5 patients were males, and 3 were females [median age 7 (range 3 to 12) years]. One case of CLL was male and 10-year old. The leukemia was diagnosed according to the previously published guideline [4]. Fourteen healthy children were used as normal controls, 9 males and 5 females, with the median age of 7 (range 2 to 10) years.

Detective method for EBV-DNA

Sample DNA preparation

2 ml of EDTA-treated peripheral blood was taken from the patients and controls, and detected once a week. The PBMNCs were separated using Ficoll-Paque. 50μ l of DNA extraction solution was added and cells were incubated in the boiling water for 10 minutes, followed by incubation at 4℃ for 8 to 12 h to sufficiently disrupt the viral particles. After centrifugation at 10,000 r/min for 5 minutes, 2μ l of supernatant was used for the amplification reaction of FQ-PCR.

Dilution of EBV-DNA quantitative standard preparation

EBV-DNA quantified standard preparation (1×10^6copy/μ l, 50μ l/tube) was centrifuged at 6,000 r/min for several seconds, labeled as 1×10^6copy/μ l. Four sterilized 0.5ml eppendorf tubes were used. 45μ l of negative control preparation was added into each tube, labeled with 1×10^2-1×10^5copy/μ l. 5μ l solution was transferred from 1×10^6copy/μ l to the tube of 1×10^5copy/μ l, then 5μ l from 1×10^5copy/μ l to 1×10^4copy/μ l, and so on until to 1×10^2copy/μ l. All the samples were stored at -20℃. The negative control and the positive control were provided by the EBV PCR fluorescent detection kit (DA AN GENE Co. Ltd. of Zhongshan University).

Procedures for amplification reaction of FQ-PCR on Roche LightCycler

2μ l solution from the pretreated samples, quantified standard preparations (5 different concentrations) and the negative control preparations was transferred into special capillaries, and centrifuged at 8000 r/min for 2 min. All the tubes were inserted into a round chuck and stayed in an inverted position for 10 seconds. After that, the mixture was used for the amplification as described below: pre-denaturation at 93℃ for 2 min, 40 cycles of amplification at 93℃ for 5 s and at 57℃ for 45 s. Detection data were auto-saved after the reaction. The fluorescent ratio (F1/F2) was used for the calculations. The correlation coefficient (r) was required to be less than minus 0.97, close to -1.0 in the analysis. The Ct value of the negative control should not be obtained. Ct values from clinical samples were plotted on the standard curve and the copies were calculated automatically by the instrument.

Clinical Therapeutic Regimen

All ALL patients initially received prednisone sensitivity test (60mg/m^2 for a week) after the confirmation of the diagnosis. The blast cells were routinely counted from peripheral blood smear on the 8th day. In the induction remission therapy, ALL patients received VDLD regimen [(vincristine, VCR); (daunorubincin, DNA); (L-asparaginase,

L-asp); (dexamethasone, Dex)]. Bone marrow examination was performed on the 19th day after the induction remission treatment. ANLL patients received DA treatment [(daunorubincin, DNA); (cytarabine, Ara-C)] as induction remission regimen. Bone marrow examination was done on the 19th day after the induction remission treatment. The patients with EBV infection received ganciclovir treatment of 5-10mg (kg.d) for 2 weeks.

Criteria for clinical therapeutic efficacy

Criteria for clinical therapeutic efficacy were based on the previously published guideline [4].

Statistical analysis

Chi-square test and four fold table precise test were used for data analysis.

Results

Establishment of the quantitative EBV-DNA standard curve

Serially diluted quantified EBV-DNA standard preparations were used as the positive controls. Serially diluted EBV- DNA was tested using real-time PCR. The dynamical curve of PCR amplification as well as the fluorescent quantitative PCR standard curve was created at the end of the reaction. The slope of the curve was minus 3.778, and the related coefficient was minus 1.00.

The EBV infection status of 35 pediatric leukemia patients

EBV infection was detected in 8 (22.86%) out of the 35 pediatric leukemia patients. The positive rate of EBV-DNA was 26.92 % (7 / 26) in ALL with the quantity of (5.144± 6.91) × 10^5 copies/ml, and was 12.5% (1 / 8) in ANLL patients with the quantity of 4.031 × 10^3 copies/ml. No EBV-DNA was detected in CLL patients and healthy controls.

Relationship between EBV Infection and clinical value

In the peripheral blood of pediatric leukemia patients with and without EBV infection, the hemoglobin was (67.50 ± 18.91) g/L vs. (85.04± 23.83)g/L (t=1.90, P>0.05)); platelet was (57.75 ± 24.88) × 10^9 /L vs. (81.85 ± 48.69)× 10^9 (t=1.34, P>0.05); and the count of leukocytes was (144.64± 46.41) × 10^9 /L vs. (31.04 ± 60.27) × 10^9 /L (t=4.90, P <0. 001), respectively. Among the patients with EBV and without infection, 7 cases (7/8 cases, 87.5%) and 2 cases (2/27cases,7.4%) had hepatosplenomegaly of more than 5 cm ((P<0. 001).

EBV infection and clinical therapeutic efficacy

In ALL patients with EBV and without EBV infection, the rate of non-response to prednisone was 100% (7/7) and 26.32%(5/19) (P=0.001); CR rate after the induction chemotherapy was 28.57% (2/7) and 84.21% (16/19) (P=0.003). In ANLL patients with EBV and without EBV infection, the CR rate after the induction chemotherapy was 100% (1/1) and 71.42%(5/7) (P=0.05); the relapse rate was 100% (1/1) and 28.57%(2/7) (P=0. 375).

Discussion

EBV was discovered by an English virologist Epstein-Barr in 1964 during the cultivation of the lymphoblast strain that belongs to genus of malignant lymphoma B95 from African children. EBV can be spread in a wide range among crowds, and EBV infection that infects healthy individuals can be controlled by the reaction of cytoxic T lymphocytes, which is restricted by the antigenic specificity of MHC. Therefore most infected children do not show obvious symptoms, but the DNA or cDNA of the virus synthesized by the reverse transcription exists in cells in an integrated form or a ring-shaped molecular form. This causes a latent state which makes it difficult to detect the virus. That is why we could not detect any EBV-DNA in the healthy controls. When the body immunologic system decreased, the virus could be reactivated. Grogan E [6] has found a kind of protein called ZEBRA protein (Bam HI Z Epstein-Barr replication activator), which plays a key role in reactivating the EBV in human lymphocytes and can make the latent virus reactivated and replicated.

There are many test methods nowadays, but FQ-PCR is a new nucleic acid detection technology [7] which has been developed in recent years. It adopts a specific fluorescent probe on the basis of routine PCR. There is a fluorescent reporting radical and fluorescent quenching radical marked on the two ends of the probe. When the probe is unbroken, the fluorescent signal given by the fluorescent reporting radical is absorbed by the fluorescent quenching radical; during the PCR amplification process, the probe is cut off by

Taq polymerase from 5→3, making the two radicals apart. The quantity of the fluorescent probes has one-to-one correspondence to that of the target genes. In the process, FQ-PCR can detect the changes of the fluorescent signals in real-time, and automatically calculate the copies of DNA at the end of the reaction. This method is simple, rapid with high specificity, and the results are reliable.

Results of this study showed that: the rate of EBV infection, with high quantity of EBV-DNA, in 35 pediatric leukemia patients was 22.86%, which was significantly higher than that in the healthy controls. The occurrence rates of peripheral leukocytosis and hepatosplenomegaly were significantly higher in the patients with EBV infection than those without. Patients with EBV infection had poorer response to prednisone and lower complete remission rate after the induction therapy, indicating that EBV might be associated with the generation and development of ALL. The mechanism might be that when EBV infects B lymphocyte, glycoprotein (gp350/220) of its outer membrane is combined with the receptor CD21 of complement C3d on the surface of B lymphocytes, and then goes into the infected B lymphocytes through endophagocytosis mediated by the receptor[8]. The EBV infection causes the mutation or translocation of lymphocyte chromosome, which may lead to the activation and over expression of oncogene c-myc, and finally to the genesis of lymphoma [9]. Some studies show that LMP-1 may hamper the apoptosis of infected lymphocytes by regulating the apoptosis factors, such as bc1-2, c-myc, p53 and so on [10,11] to promote the proliferation of cells and result in oncogenesis. Further research needs to be done to clearly elucidate the correlation of EBV infection to the onset of pediatric ALL.

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