RESEARCH ARTICLE

Clinical Significance of Quantitative Analysis of Plasma Epstein-Barr Virus DNA in Patients of Xinjiang Uygur Nationality with Hodgkin's Lymphoma

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Abstract

Objective: To investigate the relationship between plasma EBV-DNA concentration and clinicopathologic features of Hodgkin's lymphoma cases. Methods: At first, the positive rate of plasma EBV-DNA was determined with a nested-PCR method using 45 specimens from Uygur HL patients, as well as 110 healthy people sampled as normal controls. Secondly, using fluorescent quantitative nested-PCR, EBV viral load was assessed in the EBV-DNA positive plasma samples. Then, relationships between plasma EBV viral load and clinicopathologic features of HL patients were analyzed. Results: The positive rate of plasma EBV-DNA of HL patients was significantly higher than that of normal controls (53.3% vs 26.4%, *P*=0.001). There was no significant difference about plasma EBV viral load between EBV–associated HL and EBV-DNA positive normal people (*P*=0.490). Looking at patients' characteristics, plasma EBV viral load in 10-20 years EBV–associated HL was higher than in EBV cases which were less than 10 years or more than 35 years (*P*=0.025). Furthermore, in EBV-associated HL, concentration of plasma EBV-DNA was significantly higher in advanced stage disease (stages III–IV; *P*=0.013), and with B-symptoms (P=0.020). Conclusion: EBV-DNA levels were associated with part of clinicopathologic features of cases. It was of practical use to screen HL. Further etiological studies appear warranted.

Keywords: Hodgkin's lymphoma - Uygur nationality - nested-PCR - Epstein-Barr virus p1asma viral load

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Introduction

Epstein-Barr virus (EBV) might be one of the pathogenic factors of Hodgkin's lymphoma (HL) and its expression rate has obvious geographical and ethnic differences (Massini et al., 2009). In preliminary research, paraffin tissue samples of Uygur patients with HL in Xinjiang were detected for EBV detection with in situ hybridization and it was found that positive rate was 65%. Therefore, this region could be regarded as an area with geographic advantages of HL incidence research. Detection rate of EBV-DNA in serum or plasma of patients with HL had differences between different regions and races. Detection rates of EBV in peripheral blood of patients with HL in countries in North America, Europe and other developed countries were approximately 40-60% (Jarrett et al., 2003; Gandhi et al., 2006). However, up to now, there was no research report about quantitative detection of EBV-DNA in peripheral blood of EBV positive patients with HL of Uygur in Xinjiang. Consequently, in this research, dissociative EBV-DNA in peripheral blood of Uygur patients with HL was detected with fluorescence quantitative PCR combined with nested PCR technology, which was used to explore the relationship between the amount of dissociative EBV-DNA in peripheral blood and clinical pathological features. That provided part of the clue for discussion of pathogenic factors of HL in this region. In addition, it also laid the foundation for the deep research on the clinical value of plasma EBV-DNA quantitative analysis in HL early diagnosis, prognosis and monitoring of recurrence and metastasis after treatment in the future.

Materials and Methods

Object of study

From Jan. 2011 to May 2012, peripheral blood samples of 45 Uygur patients with HL which were initially treated by Affiliated Tumor Hospital of Xinjiang Medical University and outer hospitals were collected. All the patients were diagnosed by histopathology. There were 31 male patients and 14 female patients with HL. Male to female ratio was 2.2: 1. Age of onset ranged from 4 to 70 years old, with an average of 27 years old. Among

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them, 9 cases were less than 14 years old. 34 cases were aged from 14 to 60, and only 2 cases were older than 60. 110 Uygur healthy physical examinees were regarded as control group. 2-4 ml peripheral blood samples were collected from them. EDTA was used for anticoagulation. The samples were reserved at -80°C.

Experimental method

Detection of EBV in plasma samples by nested PCR qualitative detection: Plasma DNA was extracted from Uygur patients as well as healthy physical examinees with conventional phenol - chloroform method. Nested PCR was used for qualitative detection of EBV existence. Primer 5 was used to design and synthesize 2 pairs of primers for nested PCR. The following primers were all synthesized by Shanghai Sangon biological Co., LTD. The sequences were: outside primer 1: 5'GCCTAGGGGAGACCGAAGTGA 3', outside primer 2: 3'TTGCTGGACGAGGACCCTTCT5', the amplification product was a 247bp segment; inside primer 1: 5'CCAGAGGTAAGTGGACTT3', inside primer 2: 3'GACCGGTGCCTTCTTAGG5', the amplification product was a 122bp segment. Reaction conditions and procedures of outside amplification: 95°C for 2 min, (94°C for 20s, 65°C (-0.5°C/cycle) for 40s, 72°C for 30s)×9 cycles, (94°C for 20s, 60°C for 30s, 72°C for 30s)×21 cycles, 72°C for 7min, then it was reserved at 4°C. Reaction conditions and procedures of inside amplification: PCR product of the first round was diluted by 1000-fold and then it was used as the template of the second round. 95°C for 2 min, (93°C for 30s, 50°C for 30s, 72°C for 45s)×30 cycles, 72°C for 7min, then it was reserved at 4°C. 2ul PCR product of the second round was detected with 2.0% agarose gel electrophoresis. EBV-positive Akata cell DNA was used aspositive control. Electrophoresis results were observed and photographed under UV transilluminator.

Preparation of standard substance: 2 pairs of primers were designed and synthesized for detection with nested PCR. Their sequences were: outside primer 1: 5'AGGAAGCGGGTCTATGGTTG3', outside primer 2: 3'CATGTGTCCAGGCTGTGGTT5', the amplification product was a 947bp segment; inside primer 1: 5'GCCTAGGGGAGACCGAAGTGA3', inside primer 2: 3'TTGCTGGACGAGGACCCTTCT5', the amplification product was a 247bp segment. Reaction conditions and procedures of outside amplification: 95°Cfor 2 min, (94°C for 30 s, 62°C for 30 s (-0.5°C/cycle, 72°C for 2 min)×11 cycles, (94°C for 30 s, 56°C for 30 s, 72°C for 2 min)×29 cycles, 72°C for 7 min, then it was reserved at 4°C. Reaction conditions and procedures of inside amplification: PCR product of the first round was diluted with water by 1000-fold and then it was used as the template of the second round. 95°C for 2 min, (94°C for 15s, 60°C for 30 s)×40 cycles, 72°C for 7min, then it was reserved at 4°C. PCR product of the second round was purified by column. The concentration and purity were measured with UV spectrophotometer: A260/A280=1.75, A260=0.214. The concentration of the 247bp segment was 46.7 ug/ml. Converted to copy number, the concentration was 1.725×10^{14} copies/ml. Standard substance was prepared according to that concentration. Then it was mixed with an appropriate amount of human genomic DNA standard substance (Promega, G147A) to prepare standard substance of the first concentration which contained 100 ng/ul human genomic DNA and 10000 copies/ul EBV. 5-fold trace DNA diluent was used to prepare various concentrations. HL paraffin tissue samples, which were EBV-positive (detected with ISH method) in previous experiments, were selected. Among these samples, No. 1, 2, 3, 5 and 9 were also positive in detection of EBV-DNA in peripheral blood. Each of the above samples was mixed with 1.4ul genomic DNA of karyocytein peripheral blood to prepare the template of standard substance. Upstream primer of EBV Bam HI-W was 5'AACACTCCACCACACCCAGGCACA3', downstream primer was 3'TGGGTTCTTGGCCCCCTCT GGTAG5', and the amplification product was a 116bp segment. Standard substance was regarded as template. The following quantitative primer was used for test. Total volume of RT-PCR was 20 ul, including 10 ul 2×SYBR Green I, 3 ul ddH_2O, 3 ul primer and 4 ul template (standard substance). 95°C for 2 min (93°C for 45 s, 55°C for 45 s) × 40cycles. Standard curve was made. The purity of PCR amplification product was identified with melting curve of SYBR Green I and was verified by sequencing after the clone of following genes.

Detection of samples: Genomic DNA of karyocyte in peripheral blood were extracted from samples of Uygur patients with HL and healthy physical examinee which were positive in quantitative detection of EBV. Then the amount of EBV in peripheral blood was detected in accord with 1.2.2. The sequence of upstream primer of RPP14 was 5'TCCTTTGTCCT ATCTCCACAATCTGC3', and the sequence of downstream primer was 3'GGCAAGAGAGAAGGGTCCAAGTG5'. After reaction, samples were compared with standard curve by computer for calculating the copy number of EBV gene.

Calculation principle of EBV copy number: Absolute quantification was used. EBV fragment of exact concentration (A longer fragment including the 116bp segment to be detected, located in Bam HI-W fragment) was treated as standard substance for formation of EBV detection standard curve. Human genomic DNA of exact concentration was used to form RPP14 detection standard curve. EBV target fragment and RPP14 fragment of samples were amplified with 7300 quantitative PCR amplifier. Corresponding Ct value was acquired. By comparison with corresponding standard curves, molecular numbers of EBV target fragment and RPP14 fragment of samples were known. Copy number of RPP14 gene was 2 in a diploid cell, thus the number of diploid cells was half of molecular number of RPP14 fragment. All samples were unified and expressed as the number of EBV target fragment per 10000 cells.

Statistical method

SPSS 16.0 was used for statistical calculation. Comparison of detection rate of EBV DNA in plasma between patients and healthy people was tested by χ^2 test. Comparison of copy number of EBV DNA in plasma

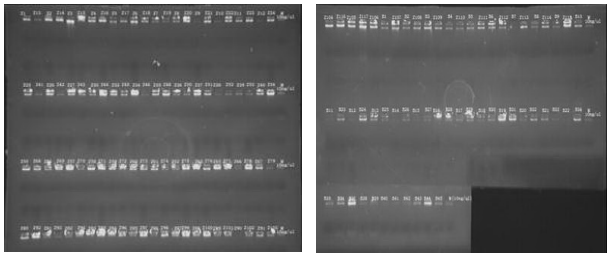

Figure 1. Gel Electrophoretograms of EBV Detection PCR of Patients Group and Control Group (healthy people)

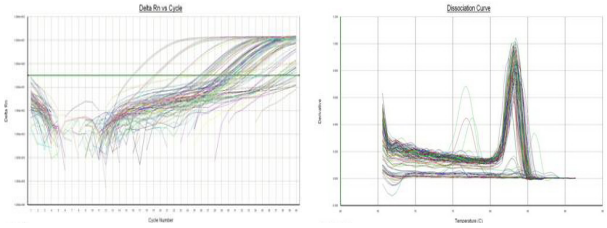

Figure 2. PCR Amplification Curve and Melting Curve of Bam HI-W Gene Fragment

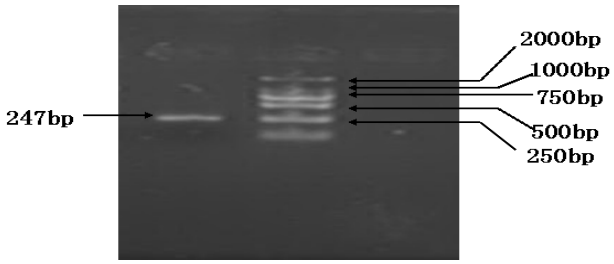

Figure 3. Electrophoretogram of Amplified 247bp EBV Bam HI-W Gene Fragment PCR

between the two groups and the relationship between copy number of EBV DNA in peripheral blood of patients and clinicopathologic factors were tested by Mann-Whitney U test.

Results

Comparison of detection rate of plasma EBV between Uygur patients with HL and healthy people

EBV detection rates of the two groups were calculated by χ^2 test. The difference of EBV detection rates between the two groups was statistically significant. EBV detection rate of patients group was higher than that of healthy people (χ^2=10.323, *P*=0.001), which was shown in Table 1. Gel electrophoretograms of the two groups were shown in Figure 1.

Formation of standard curves

The purity of PCR amplification product of EBV Bam HI-W gene fragment was identified by SYBR Green I melting curve and the result was shown in Figure 2. The electrophoretogram of amplified 247bp EBV Bam HI-W gene fragment was shown in Figure 3. In the picture, the band was correct in size and the band form was clear and the specificity of the band was good. Sequencing was shown in Figure 4. Standard curves were shown in Figure 5 and 6.

Table 1. Comparison of Detection Rates of EBV in Plasma Between Patients Group and Control Group (healthy people)

Groups	Detected cases	EBV positive	EBV negative	EBV-detection rate (%)
Patients	45	24	21	53.3
Healthy people	110	29	81	26.4
Total	155	53	102	34.2

Table 2. Comparison of EBV Molecular Numbers per 10000 Cells of the Two Groups

Groups	Detected cases	EBV molecular number per 10000 cells	
		Median	Interquartile range
Patients	24	39.34	9.11~109.34
Healthy people	29	29.51	11.14~63.72

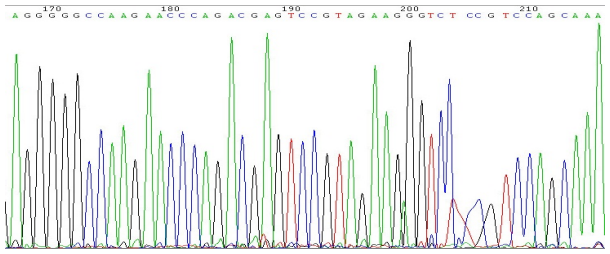

Figure 4. Sequencing Figure of 247 bp EBV Bam HI-W Gene Fragment

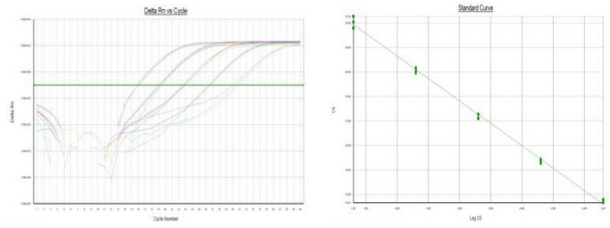

Figure 5. Standard Curve of EBV Bam HI-W Gene Fragment

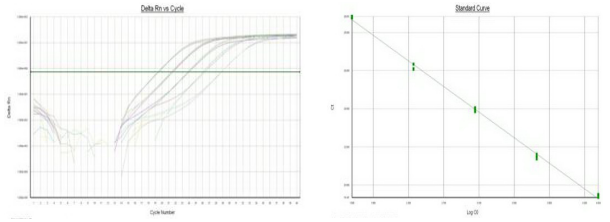

Figure 6. Standard Curve of RPP14

Quantitative detection result of EBV in plasma of Uygur patients with HL and healthy people

Samples which were positive in the EBV qualitative detection of the two groups were detected by quantitative detection of EBV-DNA. In EBV-DNA detection, the difference of EBV molecular number of 10000 cells were not statistically significant (Z=0.490, *P*=0.490). The result was shown in Table 2.

The relationship between plasma EBV-DNA quantitative detection of EBV-positive Uygur patients with HL and clinical pathological features

EBV-positive Uygur patients with HL were all with classical Hodgkin's lymphoma (cHL). The difference of EBV quantitative load of different ages was statistically

significant (P=0.025). EBV load of patients who were aged less than 10 or more than 35 was lower than that of patients aged from 10 to 20. The other differences were not statistically significant (P>0.05). EBV load of patients in stage III+IV was higher than that of patients in stage I+II and the difference was statistically significant (P=0.013). EBV load of patients without B symptom was lower than that of patients with B symptom and the difference was statistically significant (P=0.020).

Discussion

Aetiological agent of HL has not been known yet. It is shown by plenty of research reports that virus infection, such as EBV and HIV, might be one of the pathogeneses of HL.

Dissociated EBV-DNA in peripheral blood might be derived from the proliferation of tumor cells (Anker et al., 1999; Ishii et al., 2007). EBV-DNA can be detected in peripheral blood of both healthy peoples and patients. Dissociated EBV-DNA in circulating blood of patients with lymphoma are DNA fragments rather than complete virus particles (Gallagher et al., 1999; Chan et al., 2003). Currently, for different EBV DNA fragments, PCR detection efficiencies are obviously different. Bam HI-W region in EBV genome is a highly conserved and repeated sequence of EBV. Bam HI-W is a multi-copy gene. Detection of this fragment can improve sensitivity and specificity of clinical detection, thus Bam HI-W region is commonly used as target fragment of detection in clinical experiments (Le et al., 2005). Therefore, on the basis of the above features of EBV-DNA, a longer DNA sequence (247bp) containing the fragment to be detected (116bp) in EBV-DNA was directly amplified in this research. After quantification, it was directly used as standard substance. In this way, in the respects of both environment and conformation, standard substance was more close to the fragment to be detected.

Fluorescent quantitation PCR has advantages of high efficiency of nucleic acid amplification of conventional PCR, high specificity of probe technique, high sensitivity of spectrum technology and high accuracy of measurement. Initial concentration of samples can be calculated precisely by computer processing according to cycle threshold of samples and standard curves. As there were some Uygur children patients with poor cooperation, which accounted for 35.6%, blood samples collected were various in amount. The volume of blood samples ranged from 2 to 4 ml, which leaded to high requirement in detection technology. Nested PCR was a special kind of PCR. By double amplification, sensitivity and specificity of detection were effectively improved. Consequently, in this program, nested PCR was used combined with fluorescent quantitation PCR. In this way, peripheral blood samples of patients whose template contents were low could be quantitatively analyzed. This method was characterized by high sensitivity, good repeatability and good feasibility (Halliday et al., 2005). And it provided a novel approach for judging the clinical value of EBV-DNA detection in HL diagnosis, therapeutic evaluation, recurrence monitoring and prognosis.

In 2006, Musacchio (Musacchio et al., 2006) detected the situation of EBV-DNA in plasma of 30 patients with HL and found that positive rate of patients was much higher than that of healthy people (43% vs 8%, P= 0.03). Spacek et al. (2011) detected EBV infection situation of 150 patients with HL by ISH method and found 29 cases were EBV-positive (19%). Then they detected EBV-DNA in plasma and whole blood by RT-PCR. The positive rates were 76% (22/29) and 66% (19/29), respectively. That suggested that detection situation of EBV-DNA in peripheral blood was closely related with EBV positive expression in tissues. It was also indicated in this research that dissociated EBV-DNA load in plasma might be valuable for prognosis evaluation of patients with HL and follow-up visit. Positive situation of EBV-DNA in peripheral blood of Uygur patients with HL was compared with that of Uygur healthy people for the first time in this research. EBV-DNA in plasma of Uygur patients with HL was more than that of healthy people (P=0.001). That was consistent with the above literature. However, detection results of EBV-DNA in peripheral blood of patients and healthy people were different from literature at home and abroad. The reason might be that different sample sources, different target genes of detection and different detection departments could affect the detection result of dissociated EBV-DNA (Umetani et al., 2006). In this research, only one peripheral blood sample of HL patients was positive in the detection with nested PCR and negative in quantitative detection, which suggested that its specificity was high. HL tissues were detected in early experiment and 65% (26/40) of them contained EBV-DNA. EBV-DNA in corresponding peripheral blood was detected by nested PCR. Except 2 positive results, 24 cases had positive expression, suggested that detection of EBV-DNA in plasma had relatively high sensitivity. EBV-DNA in plasma might be derived from tumor cells and reflect EBV infection situation of tumor tissues. There were still different views about the role played by EBV in the HL occurrence. It was discovered in some researches that EBV infection existed before tumor occurrence and played an important role in startup of tumor occurrence. It was also believed that EBV infection only played a role in promoting the growth of tumor or was merely a kind of epiphenomenon (Chabay et al., 2008). It was found in this research that the comparison of EBV-DNA load in peripheral blood between Uygur patients with HL and healthy people was statistically significant (P>0.05). It was concluded that high tumorigenic EBV subtypes or variation existed in Uygur patients with HL and the quantity did not play a key role. Or the difference of endogenous genetic susceptibility of patients with HL was involved (Huang et al., 2012).

Children HL was closely related with EBV infection (Zhou et al., 2001; Pahand et al., 2011). In this research, it was shown that EBV-DNA load in peripheral blood of children patients aged from 10 to 20 and young adult patients was higher, which was different from some researches of western countries. In their researches, EBV-DNA load of patients with HL aged more than 50 was higher than that of young patients (Spacek et al., 2011). In addition, EBV-DNA was rarely detected in plasma or

serum of healthy and non-immunosuppressive children or adults in developed countries (Wagner et al., 2001). According to the above analyses, EBV-DNA in peripheral blood had two distributions of different ages. The reason might be that immune response of elderly patients to latent infection was low and/or reactivation of the virus increased in some developed countries. In developing countries, people tended to be exposed in EBV infection and then diseases occurred after a certain time (Wagner et al., 2001). It was shown in most researches that copy number of dissociated EBV-DNA in plasma of patients with HL was related to HL tumor staging. Musacchio et al. (2006) found that dissociated EBV-DNA in circulation was highly related to HL in progressive stage. The content of EBV-DNA in plasma of patients with HL in progressive stage was higher than that of patients in early stage. Similar results had also been observed in researches of nasopharynx cancer (Lo et al., 1999). It was also shown in this research that median copy number of dissociated EBV-DNA in peripheral blood of patients in stage III+IV (Ann Arbor staging) was obviously higher than that of patients in stage I+II (P=0.013). It suggested that copy number of dissociated EBV-DNA in peripheral blood of patients with HL not only was related to AnnArbor staging, but also accurately reflected the load of tumor as AnnArbor staging. EBV-DNA level of Uygur patients with HL was closely related to disease process. Hohaus et al. (2011) researched 18 cases of HL and found that if patients with EBV-related HL had B symptom, median load of dissociated EBV-DNA in plasma could reach14380 copies/ml. While the copy median of EBV-DNA of patients without this symptom was 0 (P=0.001). Similarly, it was shown in this research that continuous B symptom was correlated with EBV-DNA load in plasma (P=0.02).

EBV positive rate of MC in developing country was 70%-90%, followed by NS type (Dinand et al., 2006; Huang et al., 2011). However, in this research, the difference of EBV-DNA load between this two tissue types and other types was not significant (P>0.05). The correlation between MC and EBV was also reflected in distribution of age. MC type was more common in patients aged less than 20, followed by NS. That was in accord with the high load of EBV-DNA of patients aged in this range mentioned above. Therefore, the measurement of EBV-DNA load might be used in screening research of EBV-related HL in high-risk area.

In conclusion, the relationship between HL and EBV was affected by geographic distribution, race and other factors. In quantitative research of EBV-DNA in peripheral blood of Uygur patients with HL in Xinjiang, it was suggested that the role of EBV might not be direct and common. It was shown in stratification research that certain age-related type might be involved. However, it is required to expand the sample size and continue in-depth study to get more objective conclusions in the future.

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