EBER–1 ISH, LMP1 Immunohistochemical Tests and PCR for Detecting Epstein–Barr Virus from Hodgkin's Lymphoma Tissues in Southern China

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Abstract **Objective** To further explore the relationship between Epstein–Barr virus (EBV) and Hodgkin´s lymphoma (HL), three methods were employed to detect EBV in paraffin– embedded tissues of HL from Southern China. **Methods** 39 HL cases samples were studied by immunohistochemical methods for EBV latent membrane protein 1 (LMP1) expression, in situ hybridization(ISH) for EBV–encoded RNA (EBER)–1 and PCR for EBV BamH1 W fragment. **Results** In 39 HL cases, there were 66.7% (26/39) positive in LMP1 protein expression, which include 15 of 20 (75.0%) with lymphocyte predominant(LP) subtype, 9 of 14 (64.3%) with mixed cellularity (MC) subtype, 2 of 5 (20.0%) with nodular sclerosis(NS) subtype, and also 69.2%(27/39) of cases demonstrated EBER1– positive in EBER1 ISH. We also found that two samples were positive in LMP1 but negative in EBER1 ISH, also another three samples were EBER1–positive but LMP1–negative. The positive rate of PCR for EBV BamH1 W fragment presented 74.4% (29/39). Of the 4 major histopathologic subtypes of Hodgkin´s lymphoma, the lymphocyte predominant (LR) subtype is the most frequently EBV–associated, followed by mixed cellularity (MC) and nodular sclerosis (NS) subtypes. Our results also indicate that 100% of juvenile cases (older than 18 years old)were EBV positive compared to that of adults HL cases. **Conclusion** It can conclude from our results that PCR is the most sensitive method in detecting EBV in HL. But the disadvantage of this method is that it is not stable and cannot give information of the cellular localization. The best way to detect EBV is to combine PCR with IH or EBER ISH method.

More and more evidence links Hodgkin´s lymphoma (HL) to Epstein–Barr virus (EBV) infection [1–4]. The presence of EBV nucleic acids in affected tissues in HL is testified by using a variety of techniques, including spot hybridization, in situ hybridization (ISH) and the polymerase chain reaction (PCR)[2–3]. EBV related proteins, including EBV nuclear antigen 1 (EBNA 1) and the latent membrane proteins (LMP1, LMP2a and LMP2b), have also been examined by the methods of immunohistochemical assays. As we know, the percentage of EBV positive rates of HL was varied among different studies, ranged from 20% to 70%[1], one of the important reasons is partly dependent on the sensitivity of the methods employed. In order to get an accurate number of the EBV infection cases in 39 cases of paraffin embedded blocks HL, three types of methods were used in the detection.

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MATERIALS AND METHODS

MATERIALS AND SAMPLES

39 formalin –fixed and paraffin –embedded archival blocks, spanning 1987–2001, were retrieved from the departments of pathology at the following three hospitals: Nanfang hospital, which is affiliated to the First Military Medical University, Guangzhou General Hospital of PLA, and Guangzhou Children´s Hospital. All sections, diagnosed as HL previously, were re–identified by two of the authors (Zhu Mei–gang and Zhao Tong), the diagnosis of HL was established by finding of H/RS cells within an appropriate background of reactive cells, according to the criteria of the latest WHO classification[6], basing on morphology (H&E section), and immunophenotypic criteria (expression of CD20, CD30 and CD45RO antigen).

Immunohistology

Paraffin sections were stained with MAbs (from DAKO) against CD45RO antigen, CD20 antigen (L26), CD45RO antigen (UCHL1), CD15 antigen, and CD30 antigen by using the standard SP Immunohistology Kit supplied by Beijing Zhongshan Biological Company. LMP-1 was detected using a commercial cocktail of Mab against LMP1 (CS1-4, DAKO), diluted at 1:200.

Procedure was as follows briefly: 4 μm sections were mounted on slides having been coated with APES (3-aminopropyltriethoxysilane), dewaxed and endogenous peroxidase activity was blocked with 0.5% H_2O_2/methanol, transferred to 0.01 M sodium citrate solution, pH6.0, placed in a microwave oven, turned to full power, incubated for 5-8 min, processed with standard kit protocol 1 after cooling down. Finally DAB (diaminobenzidine) is used as chromogen. Known cases of EBV-positive HL were used as positive control.

EBER-1 in situ hybridization (ISH)

EBER expression was detected by using 20 base pairs of doubled digoxigenin-labelled (5′ end) oligonucleotide probes (anti-sense), 5′ CTA CAG CCA CAC ACG TCT CC 3′, designed by using Primer 5.0 software according to the EBER1 gene fragment (Gene bank gi|16326314|AB065135.1, Human herpesvirus 4 gene for EBER 1 small RNA, complete sequence). This probe was synthesized and labeled by Bioasia Biotech Company, ShangHai. The Procedure of ISH was described in the protocol of BOSD Biotech Company. Briefly, paired paraffin sections from each case were mounted on APES-treated glass slides, dewaxed and dehydrated, predigested with pepsin(3%) for 5-10 min at 30℃, pre-hybridized for 3~4 hrs and hybridized for 14-16 hrs at a concentration of 2ng/ul of probe, washed with 2×SSC for 5min×2, 0.5×SSC for 15min, 0.2×SSC for 15min at 37℃, blocked with BSA at 37℃ for 30min, treated with biotinylated-rabbit antibodies against Dig at 37℃ for 60 min, washed with 0.5M PBS for 5min×4, added SABC at 37℃ for 20 min and biotin-peroxidase at 37℃ for 20 min, then wished with 0.5M PBS for 5min×4, dyed with DAB for 10 min and counter-stained with hematoxylin for 8 min. Two known of EBV positive nasopharyngeal carcinoma cases were routinely used as controls. Two slides treated without probe were used as negative controls.

Polymerase chain reaction (PCR) techniques

DNA Preparation

DNA was extracted from formalin-fixed paraffin-embedded tissues. 7 μm thick sections were cut from each block, deparaffinized by three changes of xylene followed by ethanol washing. The samples were suspended in 50μl of digestion buffer, containing 0.25mg/ml of proteinase K (MERCK, Darmstadt, Germany), 50 mM Tris-hydrochloric acid (pH8.5), 1mM EDTA (pH8.0), and 0.5% Tween-20. Incubated at 55℃ for 3hrs, heated at 95℃ for 10 min to inactivate proteinase K, centrifuged at full speed for 10 min. The supernatant was used directly for PCR amplification. When encountered negative amplification for β-globin(house keeping gene), the DNA is purified using Qiagen columes(QIAamp DNA mini Kit, USA).

PCR procedure

Two pairs of primers were used in this study. The first pair is a house keeping gene-β-globin: PC04: 5′ CAA CTT CAT CCA CGT TCA CC 3′ and GH20: 5′ GAA GAG CCA AGG ACA GGT AC 3′, spanning 267bp. The second is designed covering 253 base pairs of EBV BamH1 W fragment based on the DNA sequences of GenBank, that is BamH 1: 5′ AAT GGG CGC CAT TTT GT 3′, BamH 2: 5′ TCC CTA GAA CTG ACA ATT 3′. The PCR reaction mixture contained 2 μl template-DNA, 2.0μl of 10×PCR buffer(containing 100mM Tris-HCl pH 9.0, 100mM KCl, 80mM $(NH4)\ SO_4$, and 0.1% NP40), 2.0mM $MgCl_2$, 400uM dNTP mixture, 10pmol each primer, and 1.5Unit Taq polymerase (Sangon, Shanghai, China) in a final volume of 20μl. The PCR programme consisted of initial incubation for 5 min at 94℃, 30 cycles of 94℃ for 30s; (55℃ for β-globin, 56℃ for BamH1 W) for 30s and 72℃ for 30s, and a final extension at 72℃ for 5 min. PCR products were visualized under short-wavelength ultraviolet-light after ethidium bromide staining of agarose gels.

Data Control

To get more accurate results, positive and negative controls were used in every test. For IHC and ISH, each case was performed two times in another laboratory. For PCR, all DNA templates were testified by using β-globin primer, only positive samples can be used to EBV amplification.

RESULTS

The clinical data, the distributions of histological subtypes and the result of EBV analysis in 39 cases of HL are shown in Table 1.

In all 39 cases, 30 males and 9 females, the sex ratio is of 3.3:1. The average age is 20.6 (range 3 to 57) years old. The adolescent patient of HL is 18(46.2%), 14 males and 4 females.

Histological sub-type

Among these cases, there were 20 cases of lymphocyte-rich type (LR), 14 cases of mixed cellularity (MC), 5 cases of nodular sclerosis (NS), and no case of lymphocyte depletion (LD) subtype. The results are summarized in Table 2.

table 1 Summary of clinicopathologic findings and ebv results

Biopsy No	age	biopsy site	Histologic type	LMP1	EBER1	PCR
1	30	cervical LN	LR	-	-	-
2	57	cervical LN	MC	-	(+)	+
3	3	cervical LN	LR	+	+	+
4	10	cervical LN	NS	+	++	+
5	10	supraclavicular LN	LR	+	+	+
6	9	cervical LN	LR	+	+	+
7	8	cervical LN	LR	+	+	+
8	36	cervical LN	NS	-	-	+
9	50	cervical LN	LR	++	++	+
10	9	cervical LN	LR	++	+	+
11	19	cervical LN	LR	-	-	+
12	18	cervical LN	LR	+	++	+
13	9	ND	MC	+++	++	+
14	20	ND	LR	-	-	-
15	49	cervical LN	LR	-	(+)	+
16	30	cervical LN	NS	-	-	-
17	8	axillary LN	MC	+	+	+
18	5	cervical LN	LR	+	+	+
19	9	cervical LN	MC	+	+	+
20	6	cervical LN	LR	+	+	+
21	5	cervical LN	LR	+	+	+
22	6	cervical LN	MC	+	+	+
23	10	cervical LN	MC	+	+	-
24	34	cervical LN	LR	+	+	+
25	40	supraclavicular LN	MC	-	-	-
26	29	femoral LN	LR	-	-	-
27	22	supraclavicular LN	MC	-	-	-
28	24	cervical LN	MC	+	+	+
29	29	cervical LN	MC	-	-	-
30	47	cervical LN	MC	+	+	+
31	28	thymus	NS	(+)	-	+
32	7	cervical LN	LR	+	+	+
33	21	supraclavicular LN	LR	(+)	-	+
34	5	cervical LN	MC	+	+	+
35	5	supraclavicular LN	MC	+	+	+
36	25	supraclavicular LN	LR	-	-	-
37	23	cervical LN	NS	-	-	-
38	28	supraclavicular LN	LR	+	++	+
39	19	cervical LN	MC	++	++	+

LR=lymphocytic predominance; MC=mixed cellularity; NS=nodular sclerosis; LN lymph node; ND: no data.

LMP1 and EBER1 expression

LMP1 positive cases were stained in the membrane and plasma of H/RS cells (Fig1.A), of which 26 cases (66.7%) showed LMP1 positive, the positive rates of LR, MC, NS subtype are 75.0% (15/20), 64.3% (9/14), and 40.0% (2/5) respectively. In contrast, the H/RS nucleuses were dyed in EBER1 ISH as described previously[3,6] (Fig1 B), in which, 27 cases (69.2%) demonstrated EBER1-positive in EBER1 ISH (Tab.2). Interestingly, among 18 LMP1-positive cases, 2 weakly LMP1-positive cases (case 31 and 33) could not be stained in the repeated EBER1 ISH attempts. Versus, another 3 EBER1-positive cases (case 2, 13, 15) showed LMP1-negative in the detection. We also find that all 18/18 (100%) cases of young patients (less than 18 years old) were both LMP1 and EBER1 positive, while only 8/21 (38.1%) adult patients were positive in LMP1 and EBER1 study. (Tab.3)

EBV gene Expression

By PCR, 29 of 39 (74.4%) cases were positive for EBV BamH1 W fragment amplification, including 16/20 cases of LR, 11/14 cases of MC and 2/5 cases of NS subtype (Tab.1, Tab.3) (Fig2). The cases (case 2, 13, 15, 31 and 33), which were identified either LMP1 or EBER1 positive, were recognized all positive with PCR. And there are two cases (case 8 and 11), both EBER and LMP1 negative, PCR positive in the detection.

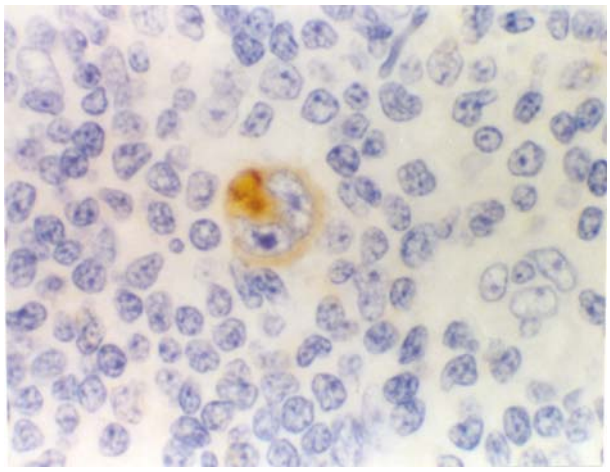

Fig.1A

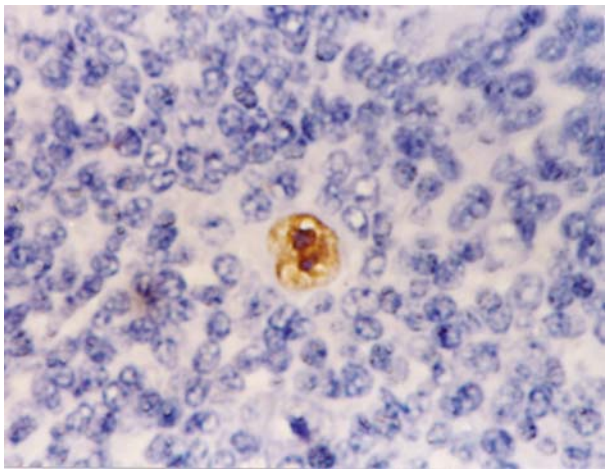

Fig.1B

Fig 1. Two cases of Hodgkin lymphoma (DAB staining with H&E counterstaining, ×1000) A. H/RS cell expressed EBV Latent membrane protein 1(LMP1); B. EBV encoded early RNA1 (EBER1).

Table 2 Comparison of the EBV positive rate with different methods in 39 cases of HL

Type of HL	Cases(n)	Positive cases (rate%)		
		LMP1	EBER1	PCR(BamH 1)
LR	20	15(75.0)	15(75.0)	16(80.0)
MC	14	9(64.3)	11(78.6)	11(35.7)
NS	5	2(40.0)	1(20.0)	2(40.0)
Total	39	26(66.7)	27(69.2)	29(74.4)

Table.3 Comparison of EBER1 and LMP1 between the young and adult HL patients

Age	EBER1(%)	LMP1(%)	total
<18	18/18(100)	18/18(100)	18
⩾18	8/21(38.1)	8/21(38.1)	21*
Total	26/39(66.7)	26/39(66.7)	39

Compared with young group, $P<0.01$

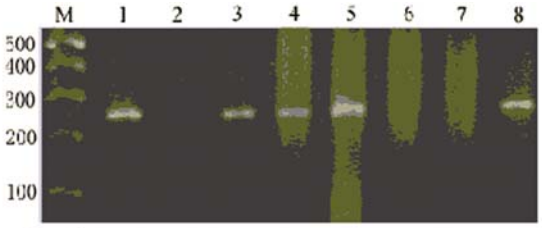

Fig 2. Result of PCR for 253 base pairs of EBV BamHI W fragment. M: marker; lane 1: positive control; lane 2: negative contorl; lane 3-8: samples: lane 3, 4, 5, 8 positive; lane 6, 7 negative.

DISCUSSION

Previous studies have shown that EBV examination may be helpful to make a correct diagnosis, expected treatment and exact prognosis for EBV associated diseases[3,8,9]. In order to compare the detection rate of the EBV detecting method, three most often used methods, ISH for EBER1 sequences, IHC for LMP1 protein and PCR for EBV BamH1 W fragments, were used to detect EBV status of 39 cases of paraffin embedded HL tissues. Our results showed that the incidence of EBV in HL is 74.4% by PCR, 69.2% by ISH for EBER1 and 66.7% by IHC for LMP1, and there is no statistic significance among them. Interestingly, for the results of EBER1 and LMP1, there are 2 (of 26) LMP1 positive cases showed EBER1 negative, another 3 (of 27) EBER1 positive cases demonstrated LMP1 negative. This result is slightly different with that of Zhou´s[6]. In his study, 17 of 27 cases of HL were LMP1 positive, 16 cases of which were EBER1 positive, only one case were slightly LMP1 positive but EBER1 negative. While our result is similar to that of Gulley´s[3].

In our experiment, we also found that most EBER1 and LMP1 expression are in all-or none manner in H/RS cells, but some cases showed only a small part of or focal H/RS cells were positive. The proper interpretation maybe some of them are destroyed during the process. As we known, EBER is RNA that is preserved in paraffin embedded tissue and easy destroyed during tissue preparation. To avoid false-negative rates, we recommend that several things had better be considered before calling LMP1 or EBER negative, Positive and negative control should be done during the experiments, all slide fields should be scanned in the diagnosis. Focal H/RS cells positive should be deemed EBV positive, it is better if EBER and LMP1 could simultaneously be done.

Compared to the other two methods, PCR technique is the most sensitive one, which is similar with the reports of other researchers[10,11]. Valente G et al [10] reported that the incidence of Hodgkin´s disease by polymerase chain reaction is 75% (39 of 52), in situ hybridization for EBER is 73.6%. Lauritzen AF et al[11] concluded that the PCR technique is the most sensitive method for detecting EBV in HD by comparison with ISH and IH staining, which is 71%, 44% and 29% respectively. Our results showed that there is no statistic significance among three methods.

EBV-relatedness also depends on age, subtype of HL, location and other characteristics of the study population[3]. Interestingly, all of our young cases (18/18, 100%) are both LMP1 and EBER1 positive. It is much higher than African cases (68%)[12], Brazilian (77%), Mexican (65%)[13]; and it is the same with results of Honduran(100%)[14] and Peruvian (100%)[15]. So our results support the view that the association of EBV with childhood HL may vary as a function of histological subtype, socioeconomic status [16,17]. Concerning Hodgkin´s lymphoma subtype, most researchers regarded that the MC subtype is most frequently EBV-associated (70%), followed by LR (50%), NS (20%) and LR (<5%) subtype[3,18]. Our results is slightly different, LR is the most frequent (75%), MC (71.4%) and NS(20%) is followed according to the result of EBER1.This difference may attribute to the choice of HL cases.

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