Int J Clin Exp Pathol 2015;8(7):7859-7868
www.ijcep.com /ISSN:1936-2625/IJCEP0010462

Original Article

Development of EBV-encoded small RNA targeted PCR to classify EBV positive diffuse large B-cell lymphoma (DLBCL) of the elderly

Jaewang Lee[1*], Min Park[2*], Min Ho Lee[2], Hyun Jun Woo[2], Hyun-Woo Kim[2], Ji Yeong Yang[2], Yong-Bin Eom[4], Sa-Hyun Kim[3], Changyoung Yoo[1], Jong-Bae Kim[2]

[1]Department of Hospital Pathology, St. Vincent's Hospital, The Catholic University of Korea, Seoul 16247, Korea; [2]Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University, Wonju 26493, Korea; [3]Department of Clinical Laboratory Science, Semyung University, Jaecheon 27136, Korea; [4]Department of Biomedical Laboratory Science, College of Medical Sciences, Soonchunhyang University, Chungnam, Asan 31538, Korea. [*]Equal contributors.

Received May 20, 2015; Accepted June 28, 2015; Epub July 1, 2015; Published July 15, 2015

Abstract: Epstein-Barr virus (EBV)-positive diffuse large B-cell lymphoma (DLBCL) of the elderly has been included in the 2008 WHO classification of lymphoma as a new provisional entity. EBV-positive DLBCL of the elderly is newly classified due to the main occurrence usually in patients of older than 50-year-old. This study was performed in 91 DLBCL patients from January 2002 to December 2012 in Catholic university of St. Vincent Hospital. Age distribution of the patients was 14~87-year-old. Specimens were collected from lymph nodes (n = 45) and extra-lymph nodes (n = 46). EBV encoded small RNA1 *in situ* hybridization (EBER1-ISH) known as a standard method for the diagnosis of DLBCL. In this study, nested PCR of DNA polymerase gene and EBER PCR were conducted to detect EBV. Presence of EBV was indicated in 3 samples (3.30%) by EBER-ISH, 26 samples (28.57%) by nPCR, and 3 samples (3.30%) by EBER PCR. The concordant results were obtained from EBER1-ISH and EBER PCR. Two samples were classified as EBV-positive DLBCL of the elderly among 91 DLBCL patients. Previously, the incidence rate of DLBCL of the elderly in Asia has been reported as 5~11%, but the result in this study showed a slightly lower incidence rate. To our knowledge, this is the first report on EBV-positive DLBCL of the elderly in Suwon area, Korea. EBER1-ISH and EBER PCR developed in this study may be helpful in classification of EBV-positive DLBCL of the elderly in future.

Keywords: Epstein-Barr virus, EBV-encoded small RNA, diffuse large B-cell lymphoma

Introduction

In 1958, Denis Burkitt first discovered a distinct type of tumor occurred in African children and reported it as a Burkitt's lymphoma [1]. In 1964, Epstein and Barr observed a malignant tumor-associated virus, which was named Epstein-Barr virus (EBV), in the lymphoma tissues by using electron microscope. EBV is a double stranded DNA virus, and infection with EBV is called a kissing disease because it is easily transmitted by physical contacts among people as well as from environment [2]. Therefore, 95% of the adult populations worldwide are infected with this virus [3]. In particular, EBV is the first virus reported that is associated with the development of cancer in human. EBV is known to play an important role in the development of various types of cancers derived from epithelial cells and lymphocytes including diffuse large B-cell lymphoma, Burkitt's lymphoma, thymic carcinoma, Hodgkin's lymphoma, gastric carcinoma and nasopharyngeal carcinoma [4-7].

EBV mainly infects B lymphocytes and epithelial cells, and once infects cells, it persists life-long asymptomatic infection [8]. There are two types of the EBV lifecycle after infection. During the lytic EBV infection, viruses are replicated and released from cells thereby causing damages to host cells as well as enabling transmission of infectious virions to other people. In the latent state of B cell infection, cell cycle, cell death

and immune response are influenced by expression of viral gene which results in the initiation of tumorigenesis and transmission of virus [9, 10]. There are three known phases of EBV latent infection characterized by the genes expressed. In latency I, Epstein-Barr nuclear antigen 1 (EBNA1) and Epstein-Barr virus-encoded small RNA (EBER) genes are expressed and Burkitt's lymphoma is associated with this latency I [11]. Further genes are expressed in latency II including latent membrane protein 1 (LMP1), latent membrane protein 2A (LMP2A), and latent membrane protein 2B (LMP2B) as well as EBNA1 and EBER. These genes are known to cause tumor, and nasopharyngeal carcinoma and Hodgkin's lymphoma are associated with latency II. In latency III, all of the 10 proteins known to be expressed in latent infection are expressed. In particular, EBER is expressed in all the phases of latent infection, and moreover EBER is highly expressed in latent infection [12, 13]. Therefore, EBER is widely used as a reliable marker for presence of EBV infection [14].

Oyama *et al.* reported lymphoproliferative disease associated with EBV, and especially in the studies on 22 patients older than 60-year-old without immune deficiency, they suggested the development of diffuse large B-cell lymphoma in association with EBV and decline in immunity in the wake of advancing in age [15, 16]. Furthermore in Korea, 380 patient samples diagnosed with diffuse large B-cell lymphoma were analyzed by EBV-encoded small RNA-1 (EBER1) *in situ* hybridization and EBV was detected in 34 of the patients. Most of the 34 patients with EBV were more than 60-year-old and showed short survival time and poor prognosis [17].

DLBCL is a B cell-derived large cell lymphoproliferative disease and it seems possible to be divided into various subtypes because of its diverse histomorphological findings, immunological, clinical and genetic feature, prognosis and response to therapy. The classification of DLBCL by WHO in 2008 became more detailed and EBV positive DLBCL of the elderly was included in the revised classification [18].

In this study, we studied 91 cases diagnosed with DLBCL at St. Vincent Hospital. The number of cases included in the EBV positive DLBCL of the elderly was investigated, and we also observed age, sex, affected organs, period of illness and mortality of the cases. In addition, we compared detection rate of standard EBER1-ISH method with PCR targeting EBER gene or nPCR targeting DNA polymerase gene.

Materials and methods

Research objects

We studied 91 cases diagnosed with DLBCL at St. Vincent Hospital from January 2002 to December 2012. Based on the reports by pathologic diagnosis, hematoxylin & eosin (H&E) stained slides that were previously prepared for diagnosis were reexamined according to the standard of WHO classification in 2008. In addition, age, sex, affected organs, period of illness and motality were also investigated.

Tissue microarray (TMA) block

To examine the samples in the same condition, we prepared TMA block. Hollow needle was used to collect tissues as small as 2 mm diameter and 3 mm depth from the sample paraffin blocks. The tissues were placed in the TMA cassette and embedded in paraffin. In total, 3 TMA blocks with 5 rows and 7 columns were produced.

In situ hybridization

To detect EBV, Epstein-Barr virus encoded small RNA-1 (EBER1)-*in situ* hybridization method was used. Four m thick sections of TMA blocks were attached to the poly-L-lysine coated slide glasses and stained by using EBER probe and ISH VIEW BLUE detection kit (Ventana medical system, Tucson, USA). The process was preceded automatically in the BenchMark XT autostainer (Ventana medical system, Tucson, USA). A tissue diagnosed as Hodgkin's lymphoma was set as an EBV positive control.

DNA extraction

Formalin-fixed paraffin-embedded (FFPE) tissues from DLBCL patients were sectioned into 10 μm thick, and three sections of each sample were placed in sterilized Eppendorf tube. Paraffin was removed by adding 1 ml xylene and the remaining xylene was removed by ethanol washing. The samples were completely dried and DNA was extracted by using DNA isolation Kit (QIAamp DNA FFPE Tissue Kit, QIAGEN Inc., Chatsworth, USA).

Table 1. Description of the primers used in this study

Primer	Primer sequence (5'-3')	Amplicon size	Gene	Reference
HVGS1	CGACTTTGCCAGCCTGTACC	225 bp	DNA polymerase gene	11
HVGS2	AGTCCGTGTCCCCGTAGATG			
EBPO1	ATGGAGAGGCAGGGAAAGAG			
EBPO1T	GGGCGTCTACCACTTTGTAA			
EBER-F1	GATCCAAACTTTAGTTTTAG	150 bp	EBV encoded small RNA 1 and 2 promoter gene	this study
EBER-R1	GCGAACCGTAACTCTATAC			

Table 2. Clinical characteristics of the DLBCL patients

Total		91
Age, yr	10~20	1
	21~30	4
	31~40	5
	41~50	16
	51~60	15
	61~70	20
	71~80	26
	81~90	4
Sex	Male	45
	Female	46
Site of biopsy	lymph node	45
	*extra-lymph node	46

*extra-lymph node: adrenal (n=1), mediastinum (n=1), brain (n=2), breast (n=2), skin (n=3), colon (n=2), femur (n=1), gingiva (n=1), ileum (n=4), jejunum (n=1), kidney (n=1), maxillary sinus (n=1), nasal cavity (n=1), occipital area (n=1), ovary (n=2), parotid gland (n=1), small intestine (n=3), stomach (n=5), testis (n=4), thyroid gland (n=1), tongue (n=1), tonsil (n=4), urinary bladder (n=2) and uterus (n=1).

PCR method for EBER gene amplification

The primers used in the experiment are listed in **Table 1**. Reaction mixture for PCR included 2 μl template DNA, 10 pmole of forward and reverse primers for EBER, 2 μl of 10 × PCR buffer, 0.2 mM deoxynucleoside triphosphate, and 0.25 U *Taq* DNA polymerase (CosmoGenetechCo., Seoul, Korea). PCR reaction was preceded in a thermal cycler (GeneAmp PCR system 2700, Perkin-Elmer Cetus, Boston, USA). Cycling conditions are as follows: denaturation at 95°C for 5 min, followed by amplification cycle at 95°C for 30 sec, 50°C 30 sec, 72°C 30 sec for 30 cycles and final extension at 72°C 7 min. Amplified product was separated on 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) for 10 min, and the image was taken by GelDoc Image Analyzer (Bio-Rad, Hercules, USA). The size of the product was compared to 100 bp DNA ladder (Fermentas, Burlington, Canada).

Production of recombinant plasmid

Recombinant plasmid was produced by using cloning kit (TOPO TA Cloning Kit, Invitrogen, Carlsbad, USA) in order for cloning of EBER gene.

Primer for EBER gene amplification

Primers for EBER gene amplification was devised using Primer 3 Input (version. 0.4.0). The sequence information is written on **Table 1**.

Sensitivity test for EBER primer

Ten-fold serial dilutions of recombinant plasmid DNA (from 1 copy/μl to 1×10^6 copies/μl) were made and used as template DNAs for sensitivity test of EBER primer. PCR reaction was conducted as previously mentioned above.

Specificity test for EBER primer

To test the specificity of the EBER primer, recombinant plasmid DNA, a sample (NO. 13) which showed positive in the PCR using EBER primer was used as a positive control and DNAs extracted from three normal lymph node specimens and three normal ileum specimens were used as a negative control. PCR reaction was conducted as previously mentioned above.

Sequencing

To confirm whether the amplified product was EBER gene, amplified products from recombinant plasmid DNA and three positive specimens (13, 37 and 69) were subjected to sequencing.

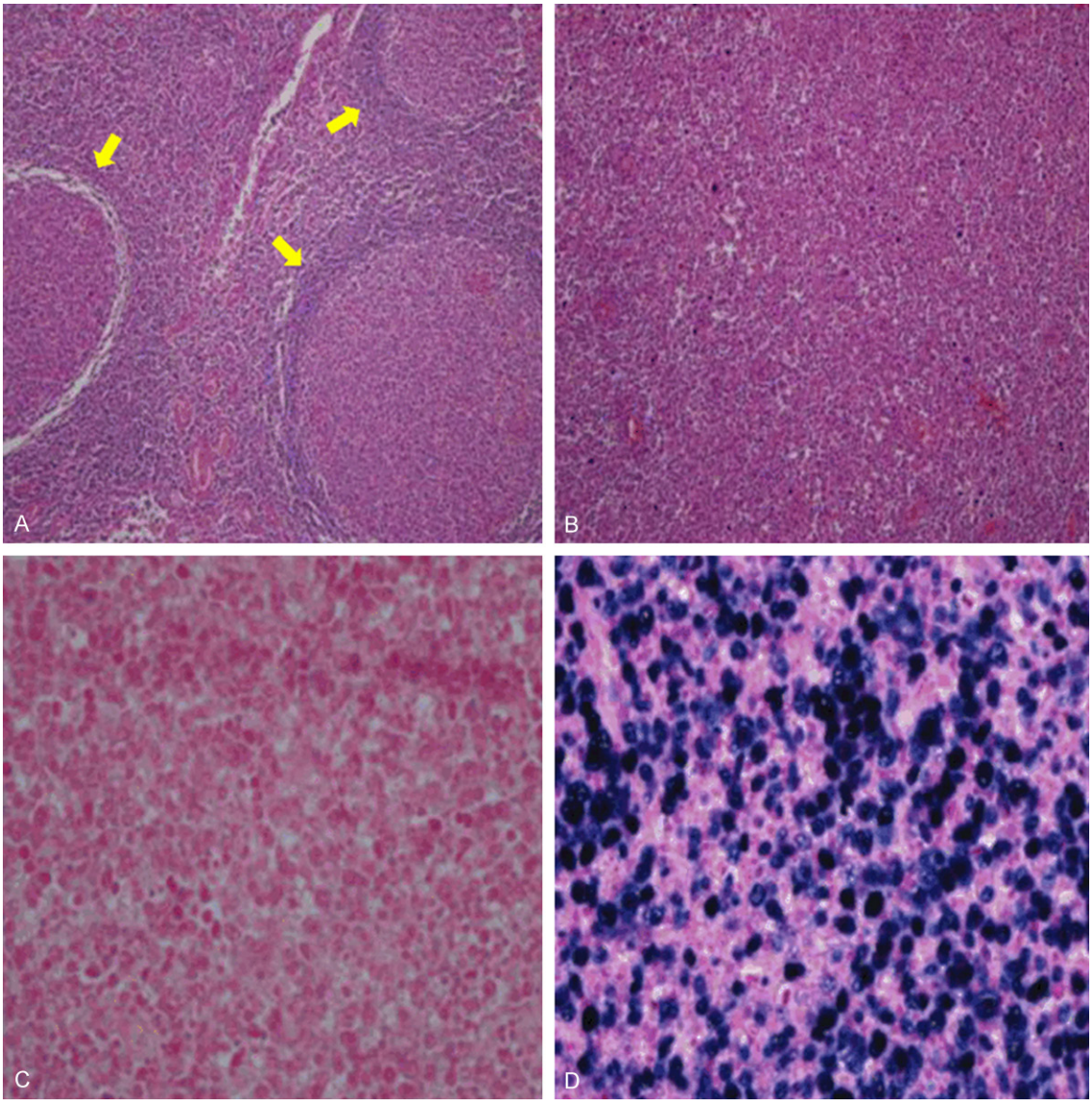

Figure 1. H&E (Hematoxylin-Eosin) stain of a DLBCL case. (A) Normal lymph node shows follicles (yellow arrows) with germinal center, mantle zone and marginal area, (B) lymph node in DLBCL patient characterized by effacement of structure without noticeable follicular structure (original magnification ×100). Representative results of Epstein-Barr virus-encoded small RNA-1 in situ hybridization. (C) Negative EBER1-ISH, (D) positive EBER1-ISH showing strong staining which indicates EBER1 transcripts of EBV accumulated in the nucleus of EBV-infected cells (Original magnification, ×400).

Nested PCR (nPCR) method for EBV DNA polymerase gene amplification

The primers used in the experiment are listed on **Table 1**. In the first step, 20 pmole of HVGS1 and HVGS2 primers were used for amplification of DNA extracted from FFPE specimen. The amplified product at the first step was used as a template DNA and 20 pmole of EBPO1 and EBPO1T were used as primers in the second step. Amplified product was separated on 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) for 10 min, and the image was taken by GelDoc Image Analyzer. The size of the product was compared to 100 bp DNA ladder.

Results

Characteristics of the patients

Ages of 91 patients diagnosed with DLBCL at St. Vincent Hospital are distributed as follows: 10- to 20-year-old (1), 21- to 30-year-old (4), 31-

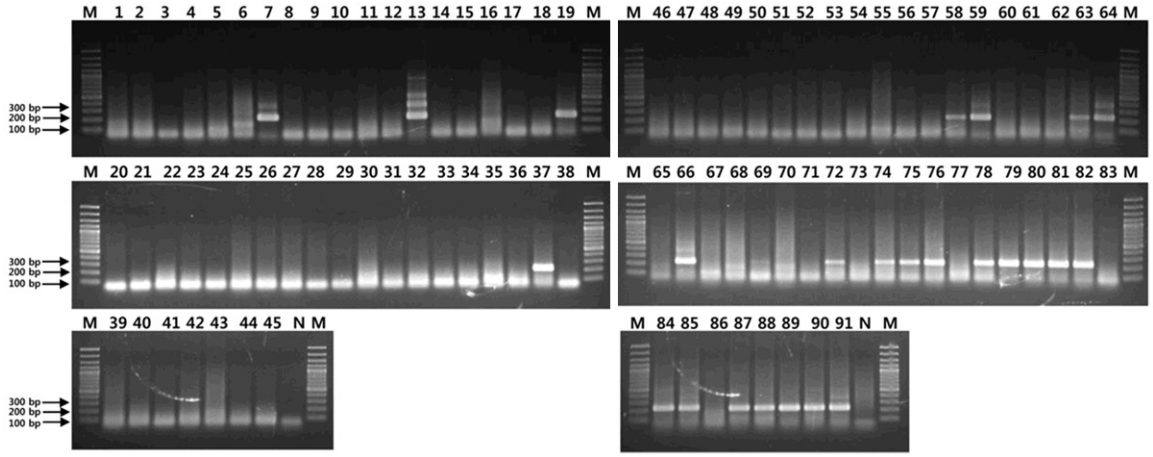

Figure 2. Nested-PCR for DNA polymerase gene of EBV from DLBCL patients. Lane M, 100 bp molecular marker; lane N, negative control; lane 1 to 91, DNAs from patients with DLBCL.

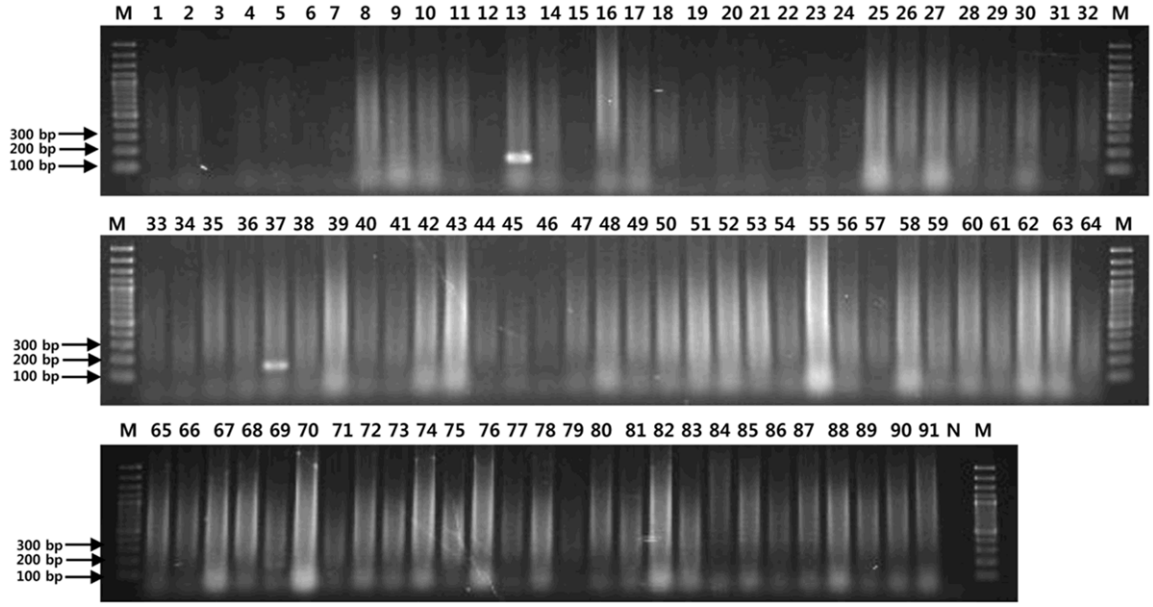

Figure 3. PCR for EBER gene of EBV from patients with DLBCL. Lane M, 100 bp molecular marker; lane N, negative control; lane 1 to 91, DNAs from patients with DLBCL; lane 13, 37 and 69 were found to be EBV positive.

to 40-year-old (5), 41- to 50-year-old (16), 51- to 60-year-old (15), 61- to 70-year-old (26) and 71- to 80-year-old (4). The age distribution was from 14- to 87-year-old, the median age was 62-year-old and the average was 59.6-year-old. The overall male and female distributions were 46 and 45 for male and female respectively (male: female =1.02:1). Specimens were collected from lymph node (45 cases) or extra-lymph node (46 cases) and the frequency of the extra lymph node was especially high at stomach, ileum, testis, tonsil, and skin (**Table 2**). According to the follow-up-survey for patients' survival, 68 of 91 cases were survived.

Result of EBER1-in situ hybridization

Of the 91 cases of DLBCL, 3 cases (3.3%) showed positive results in the EBER1-ISH method (**Figure 1C**, **1D**). In the 3 positive cases, almost all the tumor cells were positively stained. In 14 cases (15.3%), however, individu-

Table 3. Summary of clinical and PCR result of the EBV positive DLBCL of the elderly patients

NO.	Sex	Age	Diagnosis	Site	*EBER-ISH	*DNA pol	*EBER
7	M	66	DLBCL	extra-lymph node, kidney		+	
13	M	73	DLBCL	extra-lymph node, chest wall	+	+	+
19	M	65	DLBCL	extra-lymph node, tonsil		+	
37	M	49	DLBCL	lymph node, neck	+	+	+
58	M	48	DLBCL	lymph node, neck		+	
59	F	64	DLBCL	extra-lymph node, tonsil		+	
63	F	81	DLBCL	extra-lymph node, ileum		+	
64	F	42	DLBCL	extra-lymph node, breast		+	
66	F	52	DLBCL	extra-lymph node, colon		+	
69	F	74	DLBCL	lymph node, neck	+	+	+
72	F	67	DLBCL	lymph node, neck		+	
74	M	37	DLBCL	lymph node, neck		+	
75	M	14	DLBCL	lymph node, neck		+	
76	F	71	DLBCL	extra-lymph node, uterus		+	
78	F	77	DLBCL	lymph node, axillary		+	
79	F	77	DLBCL	extra-lymph node, cheek		+	
80	M	65	DLBCL	extra-lymph node, chest		+	
81	F	43	DLBCL	extra-lymph node, ovary		+	
82	F	64	DLBCL	extra-lymph node, ovary		+	
84	F	57	DLBCL	extra-lymph node, stomach		+	
85	M	42	DLBCL	extra-lymph node, ileum		+	
87	M	48	DLBCL	extra-lymph node, ileum		+	
88	M	59	DLBCL	lymph node, neck		+	
89	M	67	DLBCL	lymph node, neck		+	
90	F	69	DLBCL	lymph node, neck		+	
91	M	76	DLBCL	lymph node, neck		+	

*Abbreviations. EBER 1-ISH; EBV encoded small RNA-1, DNA pol; DNA polymerase gene, EBER; EBV-encoded small RNA gene.

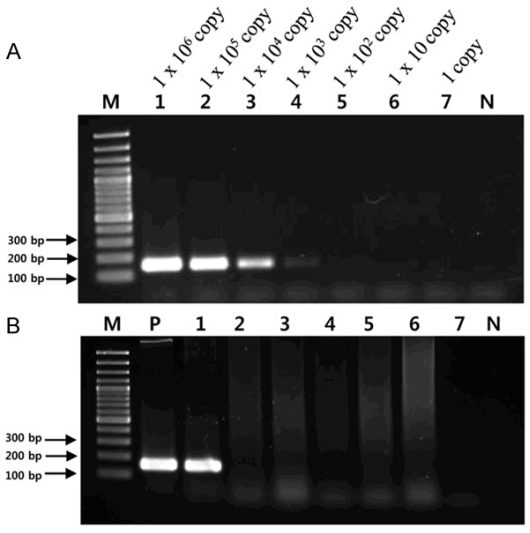

Figure 4. A. Sensitivity of EBER PCR for the detection of human herpesvirus 4 proviral DNA for EBER-1 and EBER-2 promoter. B. Specificity of EBER PCR. Lane M, 100 bp molecular marker; lane P, positive control; lane 1, ISH positive sample; lane 2 to 4, lymph node; lanes 5 to 7, ileum; lane N, negative control. Lanes 2 to 7 were ISH negative samples.

ally stained cells were dispersed in the tissue, thus we determined these cases negative.

nPCR result of EBV DNA polymerase gene

Among the 91 DLBCL cases 26 cases (28.57%) were positive for DNA polymerase gene in the nPCR results (**Figure 2**).

PCR result of EBV EBER gene

We devised primers for EBER gene investigated the 91 DLBCL cases. The result suggested that 3 of 91 cases (3.3%) were positive for EBER (**Figure 3**). The 150 bp amplified products were shown in the sample No. 13, 37 and 69 the result of which is exactly correlated to the EBER1-ISH result.

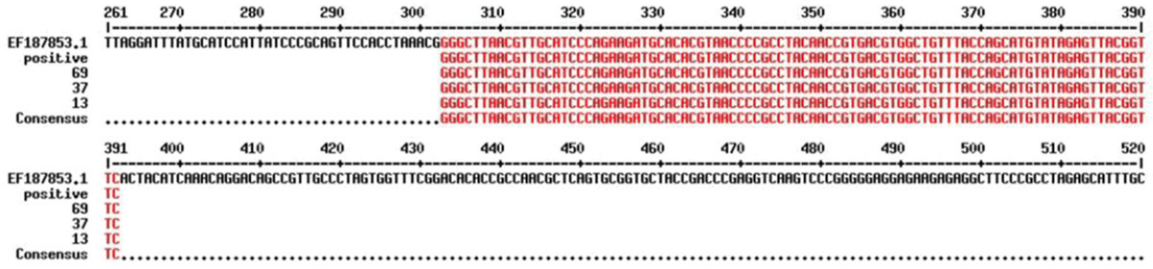

Figure 5. Comparison of the DNA sequence between EBER reference gene sequence (GenBank accession number: EF187853.1) and PCR amplicon of EBV positive samples in DLBCL patients (No. 13, 37, 69). DNA sequences of each samples and EBER reference gene sequence were same sequence.

Classification of the EBV positive DLBCL of the elderly

There were 3 cases (3.3%) positive for all the three methods (EBER1-ISH, EBER-PCR, and DNA polymerase-nPCR) among the 91 cases diagnosed with DLBCL at St. Vincent Hospital (**Table 3**). The age of each case was 47, 73 and 74 respectively, two of which were male and another of which was female. Two samples were collected from lymph node and another was from extra-lymph node (chest wall). Therefore, the two cases that were older than 50-year-old are classified as EBV positive DLBCL of the elderly.

Sensitivity and specificity of the EBER primer

Sensitivity of the EBER primer was investigated by using recombinant EBER plasmid DNA as a template DNA. Among the 10-fold diluted samples, at least 1×10^3 copy/μl sample was detected by PCR (**Figure 4A**). Furthermore, not any amplified product was detected in the normal lymph node and ileum samples but in the recombinant plasmid and positive samples which suggests the primer is specific to EBER (**Figure 4B**).

Sequencing result

Sequence of the PCR amplicon amplified by EBER primer was compared to the EBER reference gene sequence registered to GenBank (accession number: EF187853.1). PCR amplicon sequences of the positive samples (13, 37 and 69) were identical to the EBER reference gene (**Figure 5**).

Discussion

DLBCL includes various types of lymphomas with different histological findings, immunological and genetic characteristics, clinical diagnostic features, and prognosis and responsiveness to the therapy. The classification of lymphomas became more detailed in the revised WHO classification in 2008 which included EBV positive DLBCL of the elderly. EBV positive DLBCL of the elderly is closely associated with EBV and occurs in people who are older than 50-year-old [18]. In this study, we detected EBV in the specimens from DLBCL patients by using EBER1-ISH, PCR targeting EBER gene and nPCR targeting DNA polymerase gene, compared detection rate, and examined how many cases are classified as EBV positive DLBCL of the elderly.

We performed experiments based on 91 cases diagnosed with DLBCL at St. Vincent Hospital from 2002 to 2012. Previously made H&E stained slides and immunohistochemical slides were reconfirmed, and the tissues in the paraffin blocks were collected by microneedle to prepare tissue microarray block. Tissue microarray block was made to examine the samples in the same condition, and EBER1-*in situ* hybridization method was used to detect EBV in the samples. In addition, the DLBCL samples were also analyzed by nPCR and PCR methods to detect EBV.

In the results, there were three cases that were positive for all the three methods (ISH, nPCR, PCR). Among them, patients of two cases (2.4%) were older than 50-year-old thus classified as EBV positive DLBCL of the elderly. This result indicates that EBV positive DLBCL of the elderly in Korea is less frequent than in Asia (5~11%), but the frequency is closer to the frequency in Western countries (5%) [16, 17, 19]. The mechanism of EBV positive DLBCL of the elderly is still unclear, but regression of immune system caused by aging and similarity between

EBV positive DLBCL of the elderly and immune-mediated lymphoproliferative disease such as lymphoproliferative disease after transplantation somewhat explain the development of the disease [20, 21]. Number of naïve T cells decreases with advancing years, and naïve CD8+ T-cells are scarce in old people that explain debilitation of immune defense mechanism and close correlation of EBV positivity in elderly followed by proliferation of EBV+ B-cell and subsequent progression to DLBCL [22].

In our study, there was one case which was EBV positive not included in EBV positive DLBCL of the elderly because the patient was younger than 50-year-old. This result is explained by other reports of DLBCL in patients younger than 50-year-old. Beltran *et al*. reported that EBV associated DLBCL can occur in young patients thus they suggested that the age criteria or name of category should be revised [23]. Oyama *et al*. used term 'senile' rather than 'age-related' in their report regarding 22 patients with EBV+ B-cell lymphoproliferative disease because the disease could develop in young patients, and in their report in 2007, their study targeted patients more than 40-year-old [15, 16].

In the preliminary studies, it was considered that EBV invades into extra-lymph node region in most EBV positive DLBCL of the elderly patients [15, 17]. However, currently it has been reported that invasion to lymph node accounts for 70% and to extra-lymph node accounts for 30% in general. In our study, the location of the disease occurrence was one in the lymph node and another in the extra-lymph node out of 2 cases of EBV positive DLBCL of the elderly. The extra-lymph node location was skin which is one of the major extra-lymph node region invaded by EBV (skin, pharynx, tonsil, pleura, stomach, liver, spleen, appendix, bone marrow and nasal cavity) according to the reports [19, 23].

ISH has been generally used as a standard method to detect EBV associated with tumors, but several reports suggested that ISH shows negative while molecular diagnostic or immunohistochemical method shows positive which confuse the diagnosis [24-29]. In the study to detect EBV in plasma and paraffin embedded tissue conducted by Ryan *et al.*, ISH method and quantitative real-time PCR for five EBV genes including EBNA1, LMP1 and LMP2 were compared. According to their report, the real-time PCR result for EBNA1 was comparably similar to ISH result [30]. In this study, we compared three different methods which are EBER1-ISH method, previously reported nPCR method, and PCR method using specific primer for EBER devised in this study. In the results, 3 cases for ISH (3.30%), 26 cases for nPCR (28.57%) and 3 cases for PCR (3.30%) were shown to be positive for EBV. Nine-fold higher positivity was shown in nPCR results compared to ISH and PCR results. Nested-PCR generally shows higher sensitivity than conventional PCR method, and almost all people are latently infected with EBV. Therefore, we consider the higher positivity in nPCR result is due to the false-positive. All the three positive cases of ISH coincide with the positive cases of PCR which indicates exactly the same result to the standard ISH method was shown in the PCR result using EBER specific primer devised in this study. We tested the sensitivity of PCR using EBER primer to exclude false-positivity, and specificity of the primer was also evaluated. Although more cases should be evaluated because the number of EBV positive cases examined in this study was only 3 out of 91 cases, if PCR method substitute for ISH method, a large number of samples can be processed at the same time, it will be cost-effective and easy to evaluate the results.

This is the first report regarding EBV positive DLBCL of the elderly in Suwon, Korea. We expect that these data will provide the foundation to classify EBV positive DLBCL of the elderly. Furthermore, it seems necessary to investigate more cases in combination with evaluation of pathological characteristics for better understanding in EBV infection and DLBCL.

Conclusion

In this study, we compared detection rate of EBV by three different methods. Among the 91 cases, 3 cases showed positive in EBER1-ISH (3.30%), the same 3 cases were positive in EBER1-PCR (3.30%) and 26 cases were positive in nPCR (28.57%). It is notable that the PCR result using EBER specific primer devised in this study showed exactly the same result to the ISH result. Two cases (2.4%) of the three EBV positive cases were older than 50-year-old

thus classified as EBV positive DLBCL of the elderly which is less frequent than in Asia (5~11%), but the frequency is closer to the frequency in Western countries (5%). Although more cases should be evaluated because the number of EBV positive cases examined in this study was only 3 out of 91 cases, if PCR method substitute for ISH method, a large number of samples can be processed at the same time, it will be cost-effective and easy to evaluate the results.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jong-Bae Kim, Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University, Wonju 26493, Republic of Korea. Tel: +82-33-760-2423; Fax: +82-33-760-2561; E-mail: kimjb70@yonsei.ac.kr; Dr. Changyoung Yoo, Department of Hospital Pathology, St. Vincent's Hospital, The Catholic University of Korea, Seoul 16247, Republic of Korea. Tel: +82-31-249-7646; Fax: +82-31-244-6786; E-mail: ppgg2@catholic.ac.kr

References

[1] Gaio E, Amado V, Rangel L, Huang W, Storck R and Melo-Silva CA. Levofloxacin decreased chest wall mechanical inhomogeneities and airway and vascular remodeling in rats with induced hepatopulmonary syndrome. Respir Physiol Neurobiol 2013; 189: 565-570.

[2] Hayashi I, Inoue M and Hashimoto H. Nationwide investigation in Japan of the status of MRSA infections and usefulness of arbekacin. Jpn J Antibiot 1994; 47: 595-605.

[3] Grant CW, Moran-Paul CM, Duclos SK, Guberski DL, Arreaza-Rubin G and Spain LM. Testing agents for prevention or reversal of type 1 diabetes in rodents. PLoS One 2013; 8: e72989.

[4] Gustafsson EB, Ringberg H and Johansson PJ. MRSA in children from foreign countries adopted to Swedish families. Acta Paediatr 2007; 96: 105-108.

[5] Yoo JC, Kim JH, Ha JW, Park NS, Sohng JK, Lee JW, Park SC, Kim MS and Seong CN. Production and biological activity of laidlomycin, anti-MRSA/VRE antibiotic from Streptomyces sp. CS684. J Microbiol 2007; 45: 6-10.

[6] Joo EJ, Chung DR, Ha YE, Park SY, Kang SJ, Kim SH, Kang CI, Peck KR, Lee NY, Ko KS and Song JH. Community-associated Panton-Valentine leukocidin-negative meticillin-resistant Staphylococcus aureus clone (ST72-MRSA-IV) causing healthcare-associated pneumonia and surgical site infection in Korea. J Hosp Infect 2012; 81: 149-155.

[7] Kim YC, Kim MH, Song JE, Ahn JY, Oh DH, Kweon OM, Lee D, Kim SB, Kim HW, Jeong SJ, Ku NS, Han SH, Park ES, Yong D, Song YG, Lee K, Kim JM and Choi JY. Trend of methicillin-resistant Staphylococcus aureus (MRSA) bacteremia in an institution with a high rate of MRSA after the reinforcement of antibiotic stewardship and hand hygiene. Am J Infect Control 2013; 41: e39-43.

[8] Joshi MC, Magnan D, Montminy TP, Lies M, Stepankiw N and Bates D. Regulation of sister chromosome cohesion by the replication fork tracking protein SeqA. PLoS Genet 2013; 9: e1003673.

[9] Abbas Bukhari SN, Jantan I and Seyed MA. Effects of Plants and Isolates of Celastraceae Family on Cancer Pathways. Anticancer Agents Med Chem 2015; 15: 81-93.

[10] Miles TJ, Hennessy AJ, Bax B, Brooks G, Brown BS, Brown P, Cailleau N, Chen D, Dabbs S, Davies DT, Esken JM, Giordano I, Hoover JL, Huang J, Jones GE, Sukmar SK, Spitzfaden C, Markwell RE, Minthorn EA, Rittenhouse S, Gwynn MN and Pearson ND. Novel hydroxyl tricyclics (e.g., GSK966587) as potent inhibitors of bacterial type IIA topoisomerases. Bioorg Med Chem Lett 2013; 23: 5437-5441.

[11] Zhou LL, Lin ZX, Fung KP, Cheng CH, Che CT, Zhao M, Wu SH and Zuo Z. Celastrol-induced apoptosis in human HaCaT keratinocytes involves the inhibition of NF-kappaB activity. Eur J Pharmacol 2011; 670: 399-408.

[12] Wang WB, Feng LX, Yue QX, Wu WY, Guan SH, Jiang BH, Yang M, Liu X and Guo DA. Paraptosis accompanied by autophagy and apoptosis was induced by celastrol, a natural compound with influence on proteasome, ER stress and Hsp90. J Cell Physiol 2012; 227: 2196-2206.

[13] Tozawa K, Sagawa M and Kizaki M. Quinone methide tripterine, celastrol, induces apoptosis in human myeloma cells via NF-kappaB pathway. Int J Oncol 2011; 39: 1117-1122.

[14] Wong KF, Yuan Y and Luk JM. Tripterygium wilfordii bioactive compounds as anticancer and anti-inflammatory agents. Clin Exp Pharmacol Physiol 2012; 39: 311-320.

[15] Choi BS, Kim H, Lee HJ, Sapkota K, Park SE, Kim S and Kim SJ. Celastrol from 'Thunder God Vine' protects SH-SY5Y cells through the preservation of mitochondrial function and inhibition of p38 MAPK in a rotenone model of Parkinson's disease. Neurochem Res 2014; 39: 84-96.

[16] Li GQ, Liu D, Zhang Y, Qian YY, Zhu YD, Guo SY, Sunagawa M, Hisamitsu T and Liu YQ. Anti-

invasive effects of celastrol in hypoxia-induced fibroblast-like synoviocyte through suppressing of HIF-1alpha/CXCR4 signaling pathway. Int Immunopharmacol 2013; 17: 1028-1036.
[17] Nabekura T, Hiroi T, Kawasaki T and Uwai Y. Effects of natural nuclear factor-kappa B inhibitors on anticancer drug efflux transporter human P-glycoprotein. Biomed Pharmacother 2015; 70: 140-145.
[18] Liu Z, Ma L, Wen ZS, Hu Z, Wu FQ, Li W, Liu J and Zhou GB. Cancerous inhibitor of PP2A is targeted by natural compound celastrol for degradation in non-small-cell lung cancer. Carcinogenesis 2014; 35: 905-914.
[19] Li G, Liu D, Zhang Y, Qian Y, Zhang H, Guo S, Sunagawa M, Hisamitsu T and Liu Y. Celastrol inhibits lipopolysaccharide-stimulated rheumatoid fibroblast-like synoviocyte invasion through suppression of TLR4/NF-kappaB-mediated matrix metalloproteinase-9 expression. PLoS One 2013; 8: e68905.
[20] Kang H, Lee M and Jang SW. Celastrol inhibits TGF-beta1-induced epithelial-mesenchymal transition by inhibiting Snail and regulating E-cadherin expression. Biochem Biophys Res Commun 2013; 437: 550-556.
[21] Feng L, Zhang D, Fan C, Ma C, Yang W, Meng Y, Wu W, Guan S, Jiang B, Yang M, Liu X and Guo D. ER stress-mediated apoptosis induced by celastrol in cancer cells and important role of glycogen synthase kinase-3beta in the signal network. Cell Death Dis 2013; 4: e715.
[22] Ding QH, Cheng Y, Chen WP, Zhong HM and Wang XH. Celastrol, an inhibitor of heat shock protein 90beta potently suppresses the expression of matrix metalloproteinases, inducible nitric oxide synthase and cyclooxygenase-2 in primary human osteoarthritic chondrocytes. Eur J Pharmacol 2013; 708: 1-7.
[23] Chen S, Gu C, Xu C, Zhang J, Xu Y, Ren Q, Guo M, Huang S and Chen L. Celastrol prevents cadmium-induced neuronal cell death via targeting JNK and PTEN-Akt/mTOR network. J Neurochem 2014; 128: 256-266.
[24] Lu W, Jia G, Meng X, Zhao C, Zhang L, Ren Y, Pan H and Ni Y. Beta-catenin mediates the apoptosis induction effect of celastrol in HT29 cells. Life Sci 2012; 91: 279-283.
[25] Li GQ, Zhang Y, Liu D, Qian YY, Zhang H, Guo SY, Sunagawa M, Hisamitsu T and Liu YQ. Celastrol inhibits interleukin-17A-stimulated rheumatoid fibroblast-like synoviocyte migration and invasion through suppression of NF-kappaB-mediated matrix metalloproteinase-9 expression. Int Immunopharmacol 2012; 14: 422-431.
[26] Kang SW, Kim MS, Kim HS, Kim Y, Shin D, Park JH and Kang YH. Celastrol attenuates adipokine resistin-associated matrix interaction and migration of vascular smooth muscle cells. J Cell Biochem 2013; 114: 398-408.
[27] Jantas D, Roman A, Kusmierczyk J, Lorenc-Koci E, Konieczny J, Lenda T and Lason W. The extent of neurodegeneration and neuroprotection in two chemical in vitro models related to Parkinson's disease is critically dependent on cell culture conditions. Neurotox Res 2013; 24: 41-54.
[28] Venkatesha SH, Astry B, Nanjundaiah SM, Yu H and Moudgil KD. Suppression of autoimmune arthritis by Celastrus-derived Celastrol through modulation of pro-inflammatory chemokines. Bioorg Med Chem 2012; 20: 5229-5234.
[29] Nanjundaiah SM, Venkatesha SH, Yu H, Tong L, Stains JP and Moudgil KD. Celastrus and its bioactive celastrol protect against bone damage in autoimmune arthritis by modulating osteoimmune cross-talk. J Biol Chem 2012; 287: 22216-22226.
[30] Brunt JJ, Khan S and Heikkila JJ. Sodium arsenite and cadmium chloride induction of proteasomal inhibition and HSP accumulation in Xenopus laevis A6 kidney epithelial cells. Comp Biochem Physiol C Toxicol Pharmacol 2012; 155: 307-317.