Molecular Detection of Epstein-Barr Virus (EBV) in Patients with Non-Hodgkin Lymphoma Attending the Haematology Units of Tertiary Care Hospitals in Yangon

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Non-Hodgkin Lymphoma (NHL) is one of the lymphoproliferative disorders and it is common haematological malignancy in Myanmar. A cross-sectional descriptive study involved 83 cases of Non-Hodgkin Lymphoma (NHL) attending the Haematological Units, Yangon General Hospital and North Okkalapa General and Teaching Hospital, Yangon from August 2017 to September 2018. The mean age of NHL cases was 50.15±17.07 years and 47(57%) cases were male and 36(43%) cases were female. Out of 83 cases of NHL, 34(41%) cases were type I indolent chronic lymphoma, 48(58%) cases were type II aggressive lymphoma and 1(1%) case in type III highly aggressive lymphoma. After extraction of DNA, EBNA 1 was only detected in 16/83(19.3%) cases of NHL at 262 base pair by using polymerase chain reaction (PCR). EBNA2 (300 base pair) was not found in all cases by using specific EBNA2 primer. EBV serotype 1 was found in 44% in Histological Type I NHL, 50% in Type II NHL and 6% in Type III NHL, respectively. Type I NHL was mostly in follicular lymphoma, small lymphocytic and extranodal histology types and Type II NHL was mainly in diffuse large B cell lymphoma and anaplastic large cell and Type III was mostly in plasmablastic B cell lymphoma. EBV-DNA was detected in low grade and intermediate grade of NHL in this study. EBV-DNA may be one of indicators for early diagnosis of lymphoma and prognostic markers for monitoring of treatment response by radiation and chemotherapy.

Keywords: Non-Hodgkin lymphoma (NHL), Epstein-Barr virus (EBV), Polymerase Chain Reaction (PCR)

INTRODUCTION

Global cancer rates could further increase by 50% to 15 million new cases in the year 2020, according to the World Cancer Report from the World Health Organization in 2003.[1] Non-communicable diseases (NCDs) are the leading causes of death in the world and nearly 80% of NCD deaths occur in low- and middle-income countries. It comprises of mainly cardiovascular diseases, cancers, diabetes and chronic lung diseases.[2] Lymphoid malignancy varies epidemiology and aetiology in different areas around the world. In Asians, there are higher rates of aggressive non-Hodgkin lymphoma (NHL), T-cell lymphomas and extra-nodal disease. Hodgkin Lymphoma (HL) is relatively very uncommon in Asian countries.[3] Lymphoma is one of the lympho-proliferative disorders and it is common haematological malignancy in Myanmar. Incidence of lymphoma is rising gradually year by year and 607 cases were Non-Hodgkin Lymphoma (NHL) out of total 17346 cases of malignant lymphoma in Yangon General Hospital according to Yangon Cancer Registry within seven years. It was the sixth commonest cancer in that registry for both genders but the

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female is more common than male.[4] There are many risk factors including various infections (Epstein-Barr virus (EBV), human T-cell leukemia/lymphoma virus type-1 (HTLV-1), hepatitis C virus (HCV) and *Helicobecter pylori* infections), radiation, immunodeficiency, blood transfusion, familial aggregation, genetic susceptibility and chemical exposure to pesticides solvents and diets.[3]

Hodgkin Lymphoma (HL) is a lymphoid tissue neoplasm characterized by the presence of multinucleated giant cell of B cell origin called as Reed-Stenberg cell among the numerous reactive lymphocytes. It occurs more common in male than in female. It is the commonest malignancy in young adult in Asian countries.[3] Non-Hodgkin Lymphoma (NHL) is a heterogenous group of B-cell and T-cell neoplasm that develops mainly from lymph nodes anywhere in the body. There are 36 subtypes of NHL according to World Health Organization (WHO) classification of hematopoietic and lymphoid neoplasms. The incidence of NHL has been gradually increased in developed countries 2-4% annually. It has occurred more common in median age about 54 years in Asian countries. Males are more common than females in NHL. B-cell lymphomas commonly occur around the world but T-cell lymphomas are proportionally more common in Asia than in Western countries.

The Incidence of T-cell lymphomas is 28.1% in China, 16.2% in India and 32-38% in Japan. Diffuse large B cell lymphoma (DLBCL) is the most common type of NHL, approximately one-third of total cases of NHL in the world. EBV positivity is 8-15% of DLBCL with elder patients in Asia. The common types of NHL are extra-nodal NHL 28.5-45%, T-cell lymphoma (PTCL) (unspecified) 22.4%, natural killer T-cell lymphoma (NKTCL) 22.4% and angioimmunoblastic T cell lymphoma (AITL) 17.9% and ATLL 25% in Asia. They have association with EBV and HTLV-1 infections in Asian areas. Epstein-Barr virus (EBV) is a worldwide distribution and infected in more than 80% of people over the age of 30 years. It usually occurs in childhood and adulthood. The infected persons are asymptomatic, among them a minority of infected carriers may develop spontaneously to EBV-associated lymphoma.[3] It is the first human oncogenic virus related to Hodgkin lymphoma, Non-Hodgkin lymphoma, HIV-associated lymphoma and natural killer/T-cell lymphoma. Blood transfusion and organ transplant are main routes of trans-mission for EBV infection.[5] EBV can promiscuously infect normal resting B-lymphocytes and almost always transform into proliferative blast cells exhibiting B-lymphotropic nature. EBV-infected people maintain an asymptomatic condition in their lifelong period. Over 90% of people are infected with this infection in the world. In some individuals, EBV infection has been concerned in development of some cancers and autoimmune disease.

EBV is an enveloped DNA virus-encoded approximately 100 genes and consists of two subtypes (EBV-1 and EBV-2). EBV-1 is more prevalent type and more effected in transforming infected-B cells. The worst prognosis of DLBCL is associated with EBV infection in other previous studies. EBV was detected in up to 60% of all HIV-infected lymphoma and also found in more than 95% of angioimmunoblastic T-cell lymphoma.[6] Generally, up to 40% of NHL has positive EBV infection and some NHL subgroups (diffuse large B cell lymphoma of elderly have 90% of EBV positivity in other study.[7]

Epstein-Barr virus (EBV) is a member of the *Herpesviridae* family and *Gamma herpesvirinae* subfamily. It can mainly infect in lymphocytes and epithelial cells. Latent infection occurs in B lymphocytes in circular DNA form by undergoing lytic replication in B cells and epithelial cells leading to viral reproduction. Down regulation of EBV occurrs in growth-transforming gene expression in the transformed cells. It may occur lifelong latent infection in infected memory B cells by suppression of immunogenic latent proteins and silent transcripts for EBV small RNAs (EBERs) to become escape immune recognition especially in immune-compromised cases.[8] Infectious mononucleosis and oral hairy leukoplakia (OHL) are caused by EBV. There are two genotypes such as

EBV-1 and EBV-2 distinguished by divergent gene sequences encoding the EBNA-2, 3A, 3B, and 3C proteins.[9]

The primary infection of EB virus occurs during childhood with replication of the virus in the oropharyngeal lining epithelial cells followed by a latent infection of B lymphocytes. EBV concerned malignancies have been representing 1.8% of all cancer death globally and almost 50% of EBV-related malignancies occurred in East Asia.[10] EBV-DNA was detected in 13.4% of nasopharyngeal carcinoma patients who are attending Oncology Unit of Yangon General Hospital.[11] EBV type 1 is more prevalent in population from Europe, America, China and South Asia and type 2 is less prevalent in these areas that are more observed in population of Africa and Papua New Guinean.[12]

EBV-targeted therapy may be helpful for incurable stage of nasal NK/T-cell lymphoma that remains treated with multi-agent chemotherapy and radiotherapy.[5] The study was carried out to detect the major subtypes of EBV in histologically-confirmed Non-Hodgkin Lymphoma (NHL) patients who attended the Haematology Units of Tertiary Care Hospitals in Yangon and to correlate the histological types of NHL and common subtypes of EBV in these cases.

MATERIALS AND METHODS

Sampling and data collection

A cross-sectional descriptive study involved 83 cases (47 cases (57%) were male and 36 cases (43%) were female) of Non-Hodgkin Lymphoma (NHL) attending the Clinical Haematology Department Yangon General Hospital and North Okkalapa General and Teaching Hospital, Yangon during August 2017 and September 2018. Informed consent was taken from histologically confirmed NHL cases who visited outpatient departments and some were attending these two hospitals. The three millilitres (ml) of whole blood samples from all subjects were collected with ethylenediamine tetraacetic acid (EDTA) contained tubes under aseptic condition. All clinical data including fever, site of lymph node enlargement, cycles of chemotherapy and laboratory data (histology report) were collected by using proforma. DNA extraction and molecular typing of EBV was done in Pathology Research Division. All plasma/serum samples were kept in a -20ºC freezer for DNA extraction after separation of plasma/serum by centrifugation at 3000 rpm. Detection of EBV DNA was done by polymerase chain reaction (PCR) with two sets of specific primers for EBNA-1 and EBNA-2.

In this study, 83 cases of histologically-confirmed NHL were classified according to Revised European-American Lymphoma (REAL) clinical grouping of currently recognized NHL.[13] It described mainly on three types (1) Indolent chronic lymphomas (2) Aggressive lymphoma (3) Highly aggressive acute lymphoma/leukaemia (Table 2).

DNA extraction and PCR reaction

DNA was extracted from serum or plasma of each sample by using QIAmp Blood Kit (Qiagen, Germany) according to manufacturer's protocol and amplified by using EBNA-1 and EBNA-2 primer sets. Genomic DNA (0.1-0.5 μg) was added to 25 μl of PCR mix. The reaction mix contained a final concentration of 250 μ MdNTP, 1.5 mM $MgCL_{2,}$ 0.1 μ Meach primers (EBNA-1F and EBNA-1 R) and (EBNA-2F and EBNA-2R) , and 2.5U of Taq polymerase. Primers were directed to conserve Epstein-Barr nuclear antigen (EBNA-1) and (EBNA-2). After being denatured at 94ºC for 5 minutes, samples were subjected to 40 cycles of amplification (30 seconds at 94ºC, 30 seconds at 55ºC and 5 minutes at 72ºC).

Identification of EB viral serotypes

PCR products were visualized with UV light as a single band by staining with ethidium bromide after 1.5% gel electrophoresis. Molecular marker of 100 bp was used in agarose gel running.

Gene	Primer name	Nucleic sequences	Amplicon size
EBNA-1	Forward primer	TGAATACCACCAAGAGGTG	262 bp
	Backward primer	AGTTCCTTCGTCGGTAGTC	
EBNA-2	Forward primer	TGGAAACCCGTCACTCTC	300 bp
	Backward primer	TAATGGCATAGGTGGAATG	

Ethical consideration

This study was approved by the Ethics Review Committee (ERC) of the Department of Medical Research in 2017.

RESULTS

In this study, 83 blood samples were collected from 16-80 years old histologically-proven NHL patients of both genders, forty-seven male (57%) and 36 female (43%) who were attending the Clinical Haematology Department, Yangon General Hospital and North Okkalapa General and Teaching Hospital, Yangon during August 2017 and September 2018. The mean age of NHL cases was 50.15±17.07 years. The age of youngest NHL patient was 16 years male and the oldest NPC patient was 87 years female. The commonest age group of NHL patient was 41-60 years 36/83 cases (43.3%) including 18 males and 18 females (Table 1).

Table. 1. Age and sex distributions of Non-Hodgkin Lymphoma (NHL)

Age group (year)	Male (n)	Female (n)	Total cases (n)	%
<20	7	1	8	9.6
21-40	12	2	14	16.9
41-60	18	18	36	43.3
61-80	10	14	24	29.0
>80	-	1	1	1.2
Total	47	36	83	100

Fever was not common symptom in NHL cases (24/83, 28.9% had fever) and other symptoms were found in (22/83, 26.5% cases). Signs of metastasis was detected in 29% of NHL (24/83 cases) and lymph node enlargement was not common symptom in this study (30/83 cases 36.1%). Most of the NHL patients came from Bago (46%) and others from Yangon (14.5%) and Ayeyarwaddy (12%) (Table 2).

Table 2. Demographic and clinical data of the Non-Hodgkin lymphoma cases

Demographic and clinical data	Number of patients n(%)
Age	
Mean age in years	50.15±17
Sex	
Male	47(56.6)
Female	36(43.4)
Clinical symptom (fever)	
Yes	24(28.9)
No	59(71.1)
Enlarged lymph nodes	
Yes	30(36.1)
No	53(63.9)
Metastasis	
Yes	24(28.9)
No	59(71.1)
Other symptoms (weight loss, cough & deafness)	
Yes	22(26.5)
No	61(73.5)
HIV infection	
Reactive	22(26.5)
Non-reactive	61(73.5)
Chemotherapy	
Yes	35(42)
No	48(58)
Clinical units	
YGH	43(51.8)
NOGH	40(48.2)
Residence area	
Yangon	38(46)
Bago	12(14.5)
Ayeyawaddy	10(12)
Magway	9(11)
Mandalay	4(5)
Sagine	4(5)
Other regions (Yakine, Chin, Shan, Kayin, Mon, Pyae)	6(6.5)

YGH= Yangon General Hospital, NOGH=North Okkalapa General Hospital

A total 83 cases of NPC, the commonest histological type was DLBCL 57.8% (48/83 cases) and the lowest type was small lymphocytic type of NHL 3.6% (3/83 cases). According to REAL classification, type (II) aggressive lymphoma was seen in 48 /83 cases (57.8%) as the highest type of NHL and lowest in type (III) 1/83 cases (1%) in this study (Table 3).

Table 3. Proportion of EBV DNA in different histological types and grades of NHL according to Revised European-American Lymphoma (REAL) clinical grouping of currently recognized NHL

Histological types and grades	Total cases (%)	EBV-DNA Positive (%)	EBV-DNA Negative (%)
Grade(I) Chronic indolent lymphoma	34(41)	7(20.6)	27(79.4)
- Small lymphocytic (10)			
- Follicular (14)			
- Extranodal (10)			
Grade(II) Aggressive lymphoma	48(57.8)	8(16.7)	40(83.3)
- DLBCL (34)			
- Anaplastic (14)			
Grade(III) Highly aggressive lymphoma/leukemia	1(1.2)	1(100)	0(0)
Total	83(100)	16	67

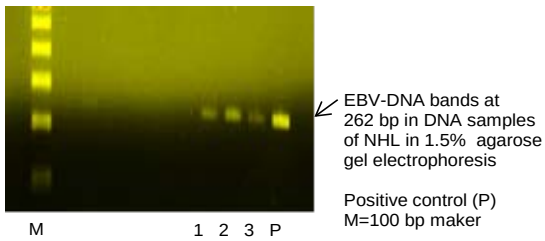

Fig. 1. Detection of EBV-DNA by using PCR with specific EBNA-1 gene

Out of 83 NHL patients, 16 patients were Epstein-Barr virus (EBV) DNA positive (19.3%) and 67 patients (80.7%) were EBV-DNA negative by amplification of polymerase chain reaction (PCR) with specific primer set of EBNA-1 gene at 262 bp. EBVNA- 2 was not detected in all NHL cases by PCR method by using specific EBVNA-2 primer. EBV-NA type 1 positive was found in 7/34 cases of Grade (I) NHL, 8/83 cases of Grade (II) NHL and 1/83 cases of Grade (III), respectively (Fig. 1).

DISCUSSION

The EB virus is one of the *Herpesviridae* families that maintain a life-long persistent infection in over 90% of healthy adult population with low copy number in memory B cells.[14] EBV Nuclear Antigen1 (EBV-NA1) is the only viral protein expressed in all forms of latent stage in EBV-related cancers. EBV genotype 1 is most prevalent in all types of lymphoma in Pakistan study.[15] Characteristic of EBV is mainly in B lymphocytes by tropism activity and it may transform to B-cell lymphoma under certain condition. The most common form of lympho-proliferative disorders related to EBV are B-cell lymphomas; Hodgkin lymphoma (HL), NHL including Burkitt lymphoma and DLBCL.[16]

In this study, most of the NHL patients participated in this research study were living in Yangon region (46%) and other regions such as Bago, Ayeyarwaddy and Magway. All NHL cases in this study have non-reactive for human immunodeficiency virus (HIV) infection and less clinical symptoms such as fever, cough, lymphnode enlarge-ment and metastatic signs and symptoms. Some EBV-positive DLBCL cases with older than 50 years show no signs of immune disorders in previous study.[16]

Forty-eight NHL cases out of 83 cases (58%) were new NHL patients who had not taken the chemotherapy and 35/83 cases (42%) were taking chemotherapy for at least 6th cycle in Haematology Units of YGH and NOGTH. The commonest age group is 41-60 years and the age range in this study was less than 18 years to more than 80 years with mean age of 50.15±17.07 years. The male population is more common than female (1.3:1) and male subjects are younger than female in less than 20 years age group (7/8) cases and the oldest 83 years was female subject in this study.

A total 83 cases of NHL, 34 cases (41%) were type (I) indolent chronic lymphoma, 48 cases (58%) were type (II) aggressive lymphoma and one case (1%) in type (III) highly aggressive lymphoma according to REAL (Revised European-American Lym-phoma) NHL classification. The most common histological type of NHL was aggressive lymphoma (grade II) especially in diffuse large B cell lymphoma (DLBCL) 34/83 cases (41%). The lowest histology type of NHL was one case/83 of precursor B lymphoblastic lymphoma in grade (III) highly aggressive lymphoma/leukemia.

DLBCL is the most common type of high-grade aggressive NHL in adults and up to 40% of prevalence rate in the world.[17] This result was similar to the findings of this study (41%). Type (I) NHL was mostly in follicular lymphoma (42%), small lymphocytic (29%) and extra-nodal histology types (29%) and type (II) NHL was mainly in diffuse large B cell lymphoma (71%) and large anaplastic cell (29%) and type (III) was mostly in plasmablastic B cell lymphoma (100%).

EBV-DNA was extracted from all serum samples of NHL cases by using QIAmp Blood Kit and EBNA1 was only detected in 16/83 cases of NHL (19.3%) in 262 base pair by using specific EBNA1 primer set with Polymerase Chain Reaction (PCR). EBNA2 (300 base pair) was not found in all cases by using specific EBNA2 primer set with PCR. EBV serotype 1 was found in 44% in histological type (I) NHL, 50% in type (II) NHL and 6% in type (III) NHL, respectively. EBV type 1 DNA was detected in 7/16 (20.6%) of grade (I) indolent chronic lymphoma (NHL), 8/16 (16.7%) in aggressive grade (II) NHL and (100%) in highly aggressive grade of NHL. In this study, EBV DNA was detected in 8/16 (50%) of DLBCL in NHL patients with more than 50 years. EBV genome was detected in 10% of DLBCL and more observed in immunocompetent patients with greater than 50 years old. In current research study, 30-70% of EBV-positive DLBCL are related to late-onset tumors depends on latencies I, II and III immune response in EBV-positive subset.[17] This finding was similar to results of current study (EBV positive in 50% of DLBCL with more than 50 years patients).

EBV serotype 1 is more prevalent in Western countries (50-75%) and Asian countries including Pakistan (75.3%) in NHL cases. (15) EBV type 1 was detected in 16/83 cases (19.3%) of NHL and lower prevalence of EBV DNA was found in NHL cases of our study. All EBV-positive samples of NHL are EBV serotype 1 and it will be carried out to genetic sequence for continuation of study.

There was not a significant correlation between histological types and grades of NHL and EBV-positive infection in this study (p=0.109) EBV-DNA was detected in low grade and intermediate grade of NHL in this study. EBV DNA may be one of indicators for early diagnosis of lymphoma and prognostic markers for monitoring of treatment response by radiation and chemotherapy. The research findings related to EBV in lymphoproliferative disorders may be provided the application of EBV targeted therapy in miRNA (micro RNA) pathway especially for NHL.

Competing interests

The authors declare that they have no competing interests.

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