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Genotypic Analysis of Epstein-Barr Virus (EBV) in Nasopharyngeal Carcinoma Patients

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Nasopharyngeal carcinoma (NPC) is endemic in certain populations, 0.6% of all cancers in the world. It occurs at high incidence in Southeast Asia, Southern China and North Africa. In Myanmar, the prevalence of NPC is gradually rising yearly and it is one of the common head and neck cancer in Yangon General Hospital (YGH). Epstein-Barr virus (EBV) is a member of the *Herpesviridae* family. It is a well-known causative agent in NPC and mainly infect in lymphocytes and epithelial cells. The polymerase chain reaction (PCR) was used to study DNA extracted from the blood samples of 35 histologically confirmed NPC patients. The commonest age group was 51-60 years in both gender and male was more common than female (1.5:1). The most common histological type was poorly differentiated squamous cell carcinoma (SCC) (45.7%) and other histological types were non-keratinized carcinoma (20%), undifferentiated anaplastic carcinoma (17.2%), moderately differentiated SCC (11.4%) and well differentiated SCC (5.7%) according to World Health Organization (WHO) classification. Primers were directed to conserved regions of the EBV genome encoding Epstein-Barr nuclear antigen 1(EBNA1) region. Specific EBV amplification (EBV-DNA positive) was found in 5 samples of NPC patients (14.3%) at 262 bp. The purified PCR products were carried out to do genetic sequencing by using ABI genetic analyzer. These isolates were found to be EB virus (*Herpesviridae* genotype 4) and firstly detected in blood samples of nasopharyngeal carcinoma patients in Myanmar. The new Myanmar EBV sequences were analyzed with a group of 14 previously published EBV strain sequences including 8 from China, 5 from Australia and one from Japan within 2006-2016. A phylogenetic tree was generated and the new Myanmar EBV strains were recorded that was different from other isolates these countries. Cancer treatment for early stage of NPC is good response but 70-80% of NPC is found in advanced or metastatic state. EBV DNA may be currently related biomarker in NPC which allows to one of the indicators for early diagnosis, better prognosis, treatment response and recurrent of disease during cancer therapy.

Key words: Nasopharyngeal carcinoma (NPC), Epstein-Barr virus (EBV), Genotypic analysis

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] Nasopharyngeal cancer (NPC) is an uncommon cancer with approximately 80,000 new cases reported per year and accounting 0.7% of all cancers. In endemic

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areas including Southern China (Hong Kong) and Southeast Asia, the annual age-standardized incidence rates are as high as 20-30 cases per 100,000 populations in men and 8-15 cases per 100,000 populations in women.[3] In Myanmar, the prevalence of NPC is 2.04% of total admission to Oto-rhinolaryngology Head and Neck Surgery Specialist Hospital, Yangon and 7.12% of total head and neck cancers in Yangon (2008-2009).[4] NPC is the second most common head and neck cancer in Yangon General Hospital (YGH) and is gradually rising the incidence yearly. According to 52 NPC cases in 2008 and 76 NPC cases in 2011, males are more common than females (2.5:1) in previous study.[5]

There are three common histological types according to the World Health Organization (WHO) such as Type (I) squamous cell carcinoma, Type 2a (II) keratinizing undifferentiated carcinoma, Type 2b (III) non-keratinizing differentiated and undifferentiated carcinoma. There were three histological grades in squamous cell carcinoma (SCC) types of NPC such as well, moderate and poorly differentiated SCC. Among them, NPC subtype (III) is the commonest form of NPC in endemic areas. Type (I) NPC is associated with Epstein-Barr virus (EBV) and sensitive to chemotherapy and radiotherapy. Type (III) NPC is also related to regulation of gene expression in DNA packaging or modulation of gene activities. Epstein-Barr virus (EBV) is a member of the *Herpesviridae* family and *Gammaherpesvirinae* subfamily. EB viral particle has a diameter of 120-180 nm and EBV genome is a linear, double stranded 172 kb DNA molecules.[6]

It can mainly infect in lymphocytes and epithelial cells. 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.[7]

The primary infection of EB virus occurs during childhood with replication of the virus in the oropharyngeal linning epithelial cells followed by a latent infection of B lymphocytes. Latent EBV infection was recognized in neoplastic cells of almost all cases of NPC. In some areas of Asia, 80% of children are infected with EBV by 6 years of age, 100% have sero-converted by 10 years of age.[8]

EBV DNA was detected in plasma/serum of 98 of 167 (58.7%) of NPC patients prior to treatment in Chulalongkorn University Hospital, Bangkok Thailand.[9] Common clinical presentations are unilateral and bilateral mass in neck, nasal obstruction with instead of bloody drainage (epistaxis) and decreased hearing.[10] The three major etiologic factors related to NPC are genetic predisposition, dietary factors including salt-cured fish and meat, foods containing aerosolizes carcinogenic nitrosamine and EB viral infection.[11]

Alcohol drinking, shrimp paste consumption and infrequent vegetables eating were found to be significantly associated to the risk of NPC in previous study from Myanmar.[12] Cancer treatment for early stage of nasopharyngeal carcinoma is good response but 70-80% of NPC is found in locally advanced or metastatic state. EB virus is currently related biomarkers in NPC that can be allowed for better prognosis, treatment response and recurrent of disease during cancer therapy.[13]

Genotypic analysis of EB virus in nasopharyngeal carcinoma patients in Myanmar was main objective of this study because the number of the NPC cases is rising gradually mainly in young adults that was mainly associated with EBV infection. Moreover, association of different histological types of NPC and genotypic characteristic of EB virus was analysed in present study. This study is provided for the strong evidence supporting the etiologic role of EBV infection in nasopharyngeal carcinoma in Myanmar.

MATERIALS AND METHODS

A cross-sectional, descriptive laboratory-based study was carried out the total 35 histologically confirmed NPC patients attending at Clinical Unit of Ear, Nose, Throat (ENT) and Head and Neck Specialist Hospital, Yangon during 2014-2016.

Blood samples of NPC patients were collected from Clinical Unit of Ear, Nose, Throat and Head and Neck Specialist Hospital, Yangon. All clinical and laboratory data were collected in proforma.

DNA extraction and molecular detection of EBV was done in Pathology Research Division and EBV DNA sequencing was carried out in Advanced Molecular Research Centre in Department of Medical Research. Thirty-five histologically confirmed new NPC cases with the age of 15-75 years and both genders were recruited. Five millilitres of whole blood with ethylene diamine tetra-acetic acid (EDTA) contained tube were collected from these subjects. All serum samples were kept in a -20ºC freezer before DNA extraction after separation of plasma/ serum by centrifugation at 3000 rpm. Detection of EBV DNA was done by polymerase chain reaction (PCR).

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 primer set. Genomic DNA (0.1-0.5 µg) was added to 25 µl of PCR mix. The reaction mix contained a final concentration of 250 µM dNTP, 1.5 mM $MgCL_2$, 0.1 µM each primers (EBNA-1F and EBNA-1 R), and 2.5 U of Taq polymerase. Primers were directed to conserve Epstein- Barr nuclear antigen (EBNA-1). 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 viral sequence

PCR products were visualized with UV light as a single band by staining with ethidium bromide after 1.5% gel electrophoresis. The PCR products were purified to remove primers and excess nucleotides by using Montage's purification method. Two micro-litres of purified product were added to a master-mix containing 3.5 µl of 5 X sequencing buffer, 0.5 µl of (Big-dye V 3.1, Applied Biosystems), 2.4 µl of sterile DDW and 1.6 µl (1 µlM concentration) of each of forward and reverse primers of EBNA gene.

Amplification was done under the following conditions: one minute of activation at 96ºC followed by 25 cycles of 10 seconds denaturation at 96ºC, 5 seconds of annealing at 50ºC and 4 minutes of extension at 60ºC. After purification of cycle sequencing product by ethanol, this product was carried out to DNA sequencing by using Applied Biosystems 3500 XL Genetic Analyzer, Hitachi.

Phylogenetic analysis

Sequence quality was checked by BioEdit software v 7.0.5 and manual correction was done. Two sequences of EBNA-1 (262nt) were compared with that of other sequences of EBV available from sequence numbers of GenBank (KX950746 and KX950747 EBNA1).

Sequence of primers used for EBNA -1 gene

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

Then, nucleotide and amino acid of all sequences were aligned by using the Cluster W 1.6 method of MEGA software version 6.0.6. Phylogenetic tree was generated by the neighbor-joining method.

Ethical consideration

This study was approved by the Ethics Review Committee of the Department of Medical Research, Ministry of Health and Sports.

RESULTS

In this study, 35 blood samples were collected from 15-75 years with both genders, 21 males (60%) and 14 females (40%) of histologically proven nasopharyngeal carcinoma patients who were attending in Clinical Unit of Ear, Nose, Throat and Head and Neck Specialist Hospital, Yangon during 2014-2015. The mean age of both genders was 47.9±14.9 years. The age of youngest NPC patient was 15 years (male) and the oldest NPC patient was 74 years (male). The commonest age group of NPC cases was 51-60 years (51.4%) including 11 males and 7 females (Table 1).

Table 1. Age and sex distributions of nasopharyngeal carcinoma

Age (Year)	Male	Female	Total cases (%)
<20	2	2	4(11.4)
21-30	1	1	2(5.7)
31-40	1	1	2(5.7)
41-50	3	2	5(14.3)
51-60	11	7	18(51.4)
>60	3	1	4(11.4)
Total	21	14	35(100.0)

Among the 35 cases of NPC, the commonest histological type was Keratinized poorly differentiated squamous cell carcinoma, 45.7% (16/35 cases) and the lowest type was Keratinized well differentiated squamous cell carcinoma 5.7% (2/35 cases) (Table 2).

Table 2. Proportion of EBV DNA in different histological types and grades of NPC according to WHO classification

Histological types and grades	Total cases (%)	EBV-DNA positive (%)	EBV-DNA negative (%)
Keratinized well differentiated SCC	2(5.7)	0(0)	2(6.7)
Keratinized moderately differentiated SCC	4(11.4)	0(0)	4(13.3)
Keratinized poorly differentiated SCC	16(45.7)	4(80)	12(40)
Non-keratinizing differentiated	7(20.0)	0(0)	7(23.3)
Non-keratinizing Un-differentiated (Anaplastic type)	6(17.1)	1(20)	5(16.7)
Total	35(100.0)	5(100)	30(100)

The commonest clinical staging was stage I (T1N0M0) NPC in 11/35 patients (31.4%) and the lowest stages was Stage 0 (lymph node is not detected) in 4/35(11.4%) in this study (Table 3).

Table 3. Clinical staging (TNM staging) of nasopharyngeal carcinoma (NPC) according to WHO classification[14]

Clinical stages	T	N	M	No. of cases (%)	EBV positive (%)
0	Tis	N0	M0	4(11.4)	0
I	T1	N0	M0	11(31.4)	1(9)
II	T1	N1	M0	3(17.1)	0
	T2	N0	M0	2(2.9)	1(50)
	T2	N1	M0	4(20)	1(25)
III	T2	N2	M0	3(11.4)	0
	T2	N2	M0	2(5.7)	0
	T3	N0	M0	1(8.6)	0
	T3	N1	M0	1(2.9)	0
	T3	N2	M0	0(0)	0
IVA	T4	N0	M0	1(8.6)	1(100)
	T4	N1	M0	1(2.9)	1(100)
	T4	N2	M0	1(2.9)	0
IVB	T any	N3	M0	1(2.9)	0
IVC	T any	N any	M1	0(0)	0
Total				35	5

T=Tumor, N=Lymph node, M=Metastasis

Of 35 NPC patients, 5 patients were Epstein-Barr virus (EBV) DNA positive (14.3%) and 30 patients (85.7%) were EBV-DNA negative by amplification of polymerase chain reaction (PCR) with specific primer set of EBNA-1 gene (Fig. 1 & Fig. 2).

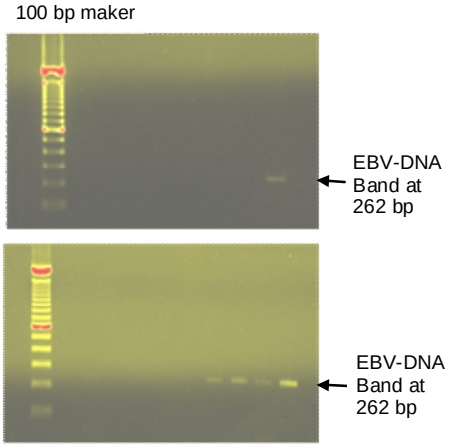

Fig.1. Detection of EBV-DNA by using PCR with specific EBNA-1 gene

EBV was detected in only one case of stage I NPC and two cases of clinical staging II and IVA NPC. This DNA was found in 16-year-old female, a case of 48 years old male, 2 males and 1 female case in the age range

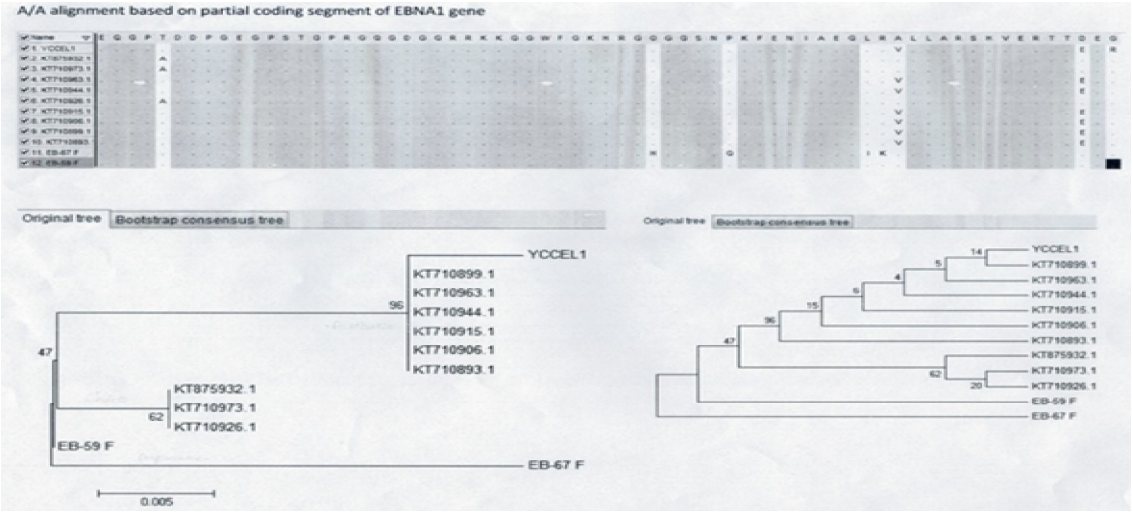

Fig. 2. Phylogenetic relationship of Epstein-Barr virus (Herpes virus genotype -4) done with EBVNA-1 gene (262 nt) analysis by Cluster W 1.6 method of MEGA software version 6.0.6. Phylogenetic tree was generated by the neighbor-joining method

of (51-60) years. EBV-DNA positivity was 4/5 cases (80%) in keratinized poorly differentiated NPC and 1/5 cases (20%) of non-keratinized undifferentiated NPC. EBV-DNA negative was found in non-keratinized differentiated type, keratinized well differentiated SCC and moderately differentiated SCC. EBV-DNA positive was detected in 2/5 cases (40%) of clinical stage I, 2/5 (20%) of Stage II and 1/5 cases (20%) of stage IV. EBV-DNA was not detected in clinical stage 0(0/4) and stage III (0/5) in this study.

Specific EBV amplification (EBV-DNA positive) was found in 5 samples of NPC patients (14.3%) at 262 bp. The purified PCR products were carried out to do genetic sequencing by using ABI genetic analyzer. These isolates were found to be EB virus (*Herpesviridae* genotype 4) and firstly detected in blood samples of nasopharyngeal carcinoma patients in Myanmar.

The new Myanmar EBV sequences were analyzed with a group of 14 previously published EBV strain sequences including 8 from China, 5 from Australia and 1 from Japan within 2006-2016. A phylogenetic tree was generated and the new Myanmar EBV strains were recorded that was different from other isolates these countries.

DISCUSSION

In this study, the commonest age group is 51-60 years in both gender and male was more common than female (1.5:1). The most common histological type was poorly differentiated squamous cell carcinoma (SCC) (45.7%). In this study, the common clinical stages were Stage I (31.4%) and stage 0(11.4%) and EBV-DNA was detected in stage I 1/11cases (9%), stage II 2/6 cases (33.3%) and Stage IVA of NPC 2/2 cases (100%), respectively. Therefore, EBV may be enhanced to progress of tumor growth.

Histologically, 4 cases of poorly differentiated type and only one case of non-keratinized undifferentiated anaplastic type were found EBV-DNA. In other study found that EB virus is commonly associated to non-keratinized lympho proliferative type of NPC. In Myanmar, the most common histological type is squamous cell carcinoma according to previous results. Therefore, 80% of EBV-DNA positive were found in poorly differentiated SCC type in this study. EBV sequence was identified in 100% of undifferentiated NPC, 50% of moderately differentiated type and 60% of keratinized NPC in a study of China.[15]

EBV has not been detected in normal nasopharyngeal epithelial cells but EBV-

DNA was detected in NPC patients and suspected NPC (carcinoma *in situ*) in other previous research findings.[16] Epstein-Barr virus is a ubiquitous herpes virus, latently infecting in all populations but primary mostly occurs in childhood with asymptomatic and acute infection in young adults, however, causes a lymphoproliferative disease called infectious mononucleosis. It also related to development of nasopharyngeal carcinoma and Burkitt's lymphoma.

It can persist for as long as ten years. There were two major types of EB virus, type1 (A) is much more B lymphocyte transformation than type 2 (B). Type 1 EBV is more common than type 2 EBV in NPC patients among Chinese and Japanese populations.[17] Type A is predominant in populations of Southern China, Japan, Tunisian, Slovenia and North America whereas type B has been found mainly in Alaska.[18]

In this study, primers were directed to conserved regions of the EBV genome encoding Epstein-Barr nuclear antigen 1(EBNA1) because this type is more commonly occur in many countries. EBV type 1 was firstly detected in 5 cases out of 35 nasopharyngeal carcinoma patients by using polymerase chain reaction and identified by genetic sequences of this virus by genetic sequencer. The new Myanmar EBV sequences were analysed with a group of 14 previously published EBV strain sequences including 8 from China, 5 from Australia and one from Japan within 2006-2016. A phylogenetic tree was generated and the new Myanmar EBV strains were recorded that was different from other isolates of these countries. Molecular detection of serum or plasma EBV-DNA could be diagnosed for early detection of NPC that was highly sensitive and specific method.

In other countries, screening of EBV-DNA could be done in patients with serially increased anti- EBV with positive family history of NPC.[19] EBV is associated with the development of NPC and EBV-DNA detection is potential for early diagnosis, monitoring and prognosis of NPC. It acts as a biomarker for better prognosis, treatment response and recurrent of disease during cancer therapy.

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