

## RESEARCH ARTICLE

# Frequency of Epstein Barr Virus Type 1 Among Nasopharyngeal Carcinomas in Iranian Patients

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### Abstract

**Background:** Around 95% of the world's population are infected with the Epstein-Barr virus (EBV), which can persist latent in B lymphocytes and epithelial cells life-long. EBV has been linked with lymphoid and epithelial cancers and persistence of EBV infection in lymphoid or epithelial cells may result in virus-associated B-cell tumors or nasopharyngeal carcinomas (NPC). This study was conducted to determine the frequency of EBV DNA in nasopharyngeal carcinoma tissue of Iranian patients. **Materials and methods:** A total of 50 blocks of formalin-fixed paraffin-embedded tissue of NPCs from 38 (76 %) male and 12 (24%) female patients were collected from archives of Ahvaz hospitals. Sections were cut at 5 µm and DNA was extracted for detection of EBV DNA and EBV typing by nested PCR. DNA sequencing was performed to confirm PCR results. The distribution of EBV DNA was compared among WHO histological subtypes of NPC. **Results:** Some 3 female and 11 (22%) male NPC samples showed positive for EBV DNA type I, 2/14(22.2%)WHO histological type II and 12/41(29.3%) WHO histological type III. **Conclusions:** The frequency of EBV DNA among NPCs in Iranian patients was found to be 28%, EBV type I predominating. Both WHO histological type II and III NPC subtypes demonstrated approximately the same detection prevalence.

**Keywords:** Epstein-Bar (EBV)- nasopharyngeal carcinoma (NCP)- nested PCR

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### Introduction

EBV is a widespread and infects about 95% of the world population. EBV can remain latent in B lymphocytes and epithelial cells and may implicate in pathogenesis of various lymphoid and epithelial malignancies, such as Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma (NPC), gastric carcinomas T cell lymphomas, post-transplant lymphoproliferative disease (Fish et al; 2014; Bieging et al., 2009; Young et al., 2004 Sharma et al., 2011; Pathmanathan et al., 1995). Epstein - Barr virus (EBV) is a member of family Herpesviridae and subfamily Gamma Herpesvirus. EBV genome is a double-stranded DNA with 184 Kilobase pairs(Kbp). Based on DNA sequence divergence in the EBNA-2 and EBNA -3 regions, the EBVs have been classified into type I and Type II (Aitken et al.,1994). Type 1 is prevalent more in china while type 2 are common in Africa and New Guinea (Sharma et al.,2011; Khabir et al.,2005). EBV is transmitted via saliva, kissing, sharing food and drinks with someone who has infectious mononucleosis. EBV has two infectivity

cycles latency, and lytic ( Sharma et al.,2011; Mirzamani et al., 2006; Markus et al.,2012). EBV DNA can persist in episomal form in the memory B-cells, and establishes latent infection by expression of EBV-nuclear antigen 1 (EBNA1) gene (Dawson et al., 2012).

Based on different EBV-associated malignancies several forms of EBV latency genes are expressed ( Temple et al., 2014; Tsao et al., 2012). In some epithelial tumors such as NPC EBV express latent membrane proteins, LMP1, LMP2A and LMP2B (Pathmanathan et al., 1995; Parikhit et al., 2016). LMP-1 is a major oncogene which activates NF-κB, JNK and p38 pathways and is critical for EBV-mediated B-cell transformation and induces Epithelial-Mesenchymal Transition (EMT) in tumor cells of NPC (Mancao et al., 2005; Izumi et al., 1999; Borthakur et al.,2016;). In epithelial cells, LMP2A is phosphorylated by csk and LMP2A constitutively activates PI3 kinase and the Akt kinase in the absence of integrin signaling. This partial substitution of integrin signaling by LMP2A leads to cell transformation and anchorage-independent growth of the HaCat keratinocyte cell line (Scholle F et al;Allen et al., 2005; Chen et al., 2005; Iwakiri 2013).

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LMP-2B impedes LMP-2A function and increases lytic activation of EBV (Rechsteiner et al., 2008; Rovedo et al., 2007). EBNA1 is the only protein expressed in all EBV-associated tumors and is critical for maintenance, replication and transcription of the EBV genome in latency (Wang et al., 2002). NPC is a unique type of head and neck squamous cell carcinoma (HNSCC), highly metastatic disease and has an uncommon malignancy in most populations of the world, with an incidence rate of one case per 100,000 person-year (Wang et al., 2013). The incidence rate of NPC in China have been reported 2.8/100,000 in men and 1.9/100,000 in women per year (Su-Mei et al., 2011). The incidence rate of NPC have been reported 0.33 per 100,000 person-year in Iran (Safavi-Naini et al., 2015). Several sensitive methods including in situ hybridization (ISH), Southern blotting and PCR (Tsuchiya et al., 2002), RNA-ISH (RISH), EBV detection (IARC., 1997) and PCR have been used for detection of EBV genome in cancer tissue (Rocio et al., 2006; Bao-Lin et al., 2012). Currently Quantification of EBV DNA load have been used in early detection of EBV in patients with nasopharyngeal cancer (Xiao-Hui et al., 2015). Since the detection of EBV has not been studied in patients with NPC in Ahvaz city, thus this study was conducted to determine the frequency of EBV DNA type 1 and 2 in patients samples with nasopharyngeal carcinoma Ahvaz city hospitals. Ahvaz city is capital of Khuzestan province with 2 million population located in the south west region of Iran.

## Materials and Methods

50 blocks formalin-fixed paraffin-embedded tissue of patients with NPC were collected from archive of Ahvaz city hospitals during 2005 to 2015. The diagnostic accuracy of nasopharyngeal carcinoma (NPC) was confirmed by a pathologist. The sections of 5 µm thickness were prepared from each sample.

### Deparaffinized tissue samples

Deparaffinization was done with xylene and ethanol (Germany, Merk). Initially, all the fragments specimens were placed in Microtubes; then, xylene was added and kept at 45°C for 15 min followed by centrifuge at 14,000rpm. This stage was repeated again. Then, the supernatant was discarded and 1ml absolute ethanol was added to precipitate and stored at the room temperature for 10 min and centrifuged at 14,000rpm for one minute. The supernatant was discarded. This process was repeated by adding 70% ethanol, same as the previous steps. Finally, supernatant was discarded and Microtubes were placed at 65°C for 5 min to vaporize the ethanol residue and the pellet was collected and used in DNA extraction (Habibian et al., 2013).

### DNA extraction

High pure PCR template preparation kit (Roche, Germany) was used for extraction of DNA, according to the manufacturer's instruction. The extracted DNA was stored at -70°C until PCR amplification.

### Nested PCR

Based on detection of EBNA-2 region of EBV genome, the typing of EBV is determined (Rocio., 2006). The nested-PCR was performed in two rounds, for first round the following primers, EBNA-2F: TGGAAACCCGTCCTACTCTC(48572-89) and anti-sense EBNA-2I: TAATGGCATAGGTGGAATG(49355-73) was used. 25 µl reaction mixture, containing 7 µl of the extracted DNA, 2.5 µl PCR buffer 10X (Roche), 0.5 µl deoxynucleotide triphosphate 10mM (Roche), 1U Taq Polymerase (Roche), 20 µM of each primer and distilled water up to 25 µl was subjected to thermocycler (Techne TC-5000, UK) and programmed for 1 cycle: 94°C for 5 min followed by 35 cycles: 94°C for 45 s, 50.5°C for 45 s, 72°C for 45 s and final extension 72°C for 10 min. The second round, 4 µl of the first round PCR product, with forward primers EBNA-2C, AGGGATGCCTGGACACAAGA (48810-29) and anti sense EBNA-2G, GCCTCGGTTGTGACAGAG (49048-65), was used for EBV type 1 detection. For type 2 determination EBNA-2B anti sense TTGAAGAGTATGTCCTAAGG was used. Following thermal condition was programmed: 1 cycle, 94°C for 5 min: followed by 35 cycles: 94°C for 45 s, 53.5°C for 45 s, 72°C for 45 s and final extension 72°C for 10 min (Rocio et al., 2006).

### Gel electrophoresis

The final PCR products were subjected to electrophoresis in 2% agarose gel, stained with safe stain and visualized under ultraviolet transilluminator. Cell line B95.8 was used as a positive control to detect EBV (29 Rocio et al., 2006). Expected PCR product for EBV type 1 was 250 bp and for EBV type 2 (300 bp) (2 Rocio et al., 2006).

### Sequencing

To determine EBV genotyping the PCR products of 14 positive samples were sequenced (ABI, Bioneer company, South Korea) and blasted using NCBI and EBV database.

### Statistics

The variable such as mean, standard deviation, were analyzed using SPSS version 20, the frequency of EBV DNA among the males and in WHO Histology Types were analyzed by chi-square and Fisher's exact tests.

## Results

38/50 (76%) of patients with Nasopharyngeal Carcinoma (NPC) were males and 12/50 (24%) females. High Frequency of 41/50 (82%) NPC were observed among the patients > 50 years while lower cases of 9/50 (18%) NPC were found among the patients < 50 years (Table 1). Among 50 NPC cases 14 (28%) showed positive for EBV DNA including 3 (6%) females and 11 (22%) males (OR = 1.222, 95% CI = 0.277 -- 5.384, p = 0.791). All the detected positive EBV DNA were belonged to EBV type 1, but EBV type 2 was detected among the patients with NPC. Fig. 1 shows the results of EBV DNA by PCR. 3/50 (6%) of females and 11/50 (22%) of males samples

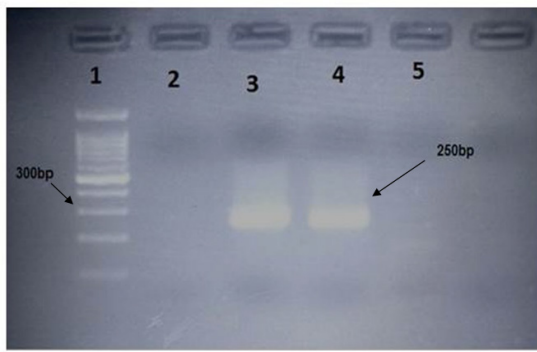


Figure 1. Shows the Results of EBNA2 Amplification Lane 1:100-bp size marker; Lane 2: Negative control; Lane 3:Positive control (positive band 250 bp (B95.8 cell line) );Lanes 4-: positive EBV DNA sample.

Table 1.Characteristics of Patients with Nasopharyngeal Carcinoma

Age group	Gender		Total
	Female	Male	
<50	3(6%)	6(12%)	9(18%)
>50	9(18%)	32(64%)	41(82%)
Total	12(24%)	38(76%)	

Table1 shows 82% of NPC patients were above 50 years while 18% were below 50 years old

NPC samples showed positive for EBVDNA type1. EBVDNA was found in 2/14(22.22%)WHO histological type II while 12/41(29.26%) of WHO histological type III showed positive for EBVDNA. ( OR= 0.690, 95% CI= 0.125-- 3.815, p = 0.670). The EBVDNA was not found in WHO histological type 1 (Table 2).

## Discussion

Appropriate diagnosis of NPC is crucial for patients' survival. The association of viral infection with nasopharyngeal cancer is important strategy for future treatment and prevention. The role of EBV in NPC is most prominent and well documented (Pathmanathan et al., 1995;Mancao et al., 2005; Izumi et al., 1999; Borthakur et al.,2016; Fish et al; 2014; Bieging et al., 2009; Allen et al., 2005; Chen et al., 2005; Iwakiri et al.,2013; Parikh et al., 2016). The detection of EBVDNA have been reported in NPC (Mirzamani., 2006; Hassan., 2006). In our study, 38(76 %) males and 12(24%) were females patients with NPC which inconsistent with Safavi-Naini et al finding in Iran (Safavi-Naini et al.,2015). With regard to a total collected 50 NPC samples during 10 years (2005-2015) , it seems low rate of nasopharyngeal carcinoma exist in our region (Safavi-Naini et al.,2015). In our study most of patients with NPC were above 50 years which is consistent with Wang et al finding (2002). Based on the EBNA2, EBNA-3a, and EBNA-3c latent genes, EBV has been classified into two major strains , which are called as EBV type 1 (EBV-1) and type 2 (EBV-2) (Rowe et al., 1989;Cancian et al., 2011; Lucchesi et al., 2008;Sample et al., 1990;Rocio et al., 2006; Dolan et al., 2006). The two strains not only differ in their genotypes, but also they have functional differences in their transforming

Table 2. Distribution of EBVDNA among Gender and WHO Histology Type.

Feature	Positive	Negative	P value
n=50			
Sex			
Male = 38	11 (28.9%)	27 (71.5%)	0.791
Female= 12	3 (25.0%)	9 (75.0%)	
WHO Histology Type			
WHO Type II =9	2 (22.2%)	7 (77.8%)	0.670
WHO Type III =41	12 (29.3%)	29 (70.7%)	

The distribution of EBV DNA among males, females, (p=791) , and in WHO histology type II, III (0.671).

capacities. EBV-1 easily transforms B cells in culture, leading to immortalized lymphoblastoid cell lines (LCL), while EBV-2 unable to transform B cells (Dolan et al., 2006; Rowe et al., 1989). Type EBV type 1 is commonly associated with NPC tumor worldwide(Klumb et al.,2004; Bao-Lin et al., 2012; Xiao-Hui et al., 2015; Habibian et al.,2013; Yap et al., 2007; Kwok et al., 2014; Parviz et al., 2014) while EBV type 2 can infect and persist latent in CD8+ T cells(Coleman et al., 2015). In our study, EBVDNA type 1 was only detected in NPC tumor and is consistent with other findings (Habibian et al., 2013; Bao-Lin et al., 2012; Xiao-Hui et al., 2015). In our survey all the NPC samples were negative for EBV DNA type 2 strain. The expression of EBNA-1, LMP -1 and LMP-2 proteins' have been exhibited to have an important role in NPC (Pathmanathan et al., 1995; Mancao et al., 2005; Izumi et al., 1999; Borthakur et al.,2016; Fish et al; 2014; Bieging et al., 2009; Allen et al., 2005; Chen et al., 2005; Iwakiri et al., 2013; Parikh et al., 2016). In our study the expressions of EBNA-1, and LMP -1 and LMP-2 were not studied but requires further investigation. EBV infection commonly detected in NPC tumors with WHO histology type II and III subtype NPC ( Chua et al 2003; Pakkirmasthan et al., 2015). In our study EBVDNA was found in 2 WHO histological type II while 12 EBV DNA were detected in WHO histological type III NPC subtypes. The prevalence of EBVDNA among 3(6 %) female and 11(22%) male patients with NPC was not found significant (P=0.791).

NPC is a complex disease and several factors such as environmental , genetic susceptibility, consumption of salted fish , preserved vegetables , cigarette smoking and family history may contribute to development of NPC(Ruan et al., 2010; Jin et al., 2016).

The concentration of plasma EBV DNA would be able to reflect the tumor load in patients with NPC (Lin et al ., 2004; Chai et al.,2012). Following surgical resection of NPC tumor has been cleared EBV DNA in plasma within short time(Chan et al.,2008), but treated NPC patients by radiotherapy/chemoradiotherapy positive for EBV DNA resulted in development of metastasis (Leung et al., 2014). The recurrence of NPC positive for EBVDNA was described in patients with post-treatment EBV (Chan et al.,2002).

In the light of mentioned data to improve treatment and management ,the screening of EBV DNA should be

implemented for patients with NPC.

In summary, the frequency of EBVDNA among the patients with NPC was 14 (28%) cases. EBV type I is dominant in NPC tumors. EBVDNA was found in 2/9 (22.22%) WHO histological type II while 12/41 (29.26%) of WHO histological type III showed positive for EBVDNA. To improve treatment and management the screening of EBV DNA should be implemented for plasma or biopsy of patient with NPC before chemotherapy.

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## Authors' Contributions

Study concept and design: ManoochehrMakvandi, Ali Teimoori, Nader saki, Soheila Nikakhlagh and Nastran Ranjbar; acquisition of data: Toran Shahani; analysis and interpretation of data: ManoochehrMakvandi and Toran shahani ; drafting of the manuscript: Toran Shahani, Zeinab Hosseini and NiloofarNeisi; critical revision of the manuscript for portant intellectual content: ManoochehrMakvandi; statistical analysis: Manoochehr Makvandi and Toran SHahani and Zeinab Hosseini; Administrative, technical, and material support: Toran Shahani, NiloofarNeisi, Ali Teimoori, Samira Pourrezaei , Zeinab Hosseini, Hashem Radmehr, AbdolnabiShabani, Hamideh Mehravaran, Azadeh hagh and, HadisKiani,; Study supervision: ManoochehrMakvandi and Ali Teimoori.

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## Financial Disclosures

Authors have no financial interest related to the material in the manuscript.

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