ORIGINAL ARTICLE

Detection of Epstein-Barr virus infections in Egyptian Nasopharyngeal Carcinoma Patients

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ABSTRACT

Background: *Epstein Barr Virus (EBV) is associated with at least 1% of global cancers including nasopharyngeal cancer (NPC). The EBV DNA importance in the prediction of NPC has received little attention in non-endemic areas.* ***Objective*** *of the current study was to investigate the frequency of EBV in NPC patients compared to control of healthy individuals, and to study the relationship between EBV infection and clinicopathological and demographic characteristics.* ***Methodology:*** *A Case control study was conducted at the Hospital of Mansoura University. Fresh biopsies were collected from NPC patients and healthy controls over 2-years period between 2021 and 2023. After previous DNA extraction, real-time polymerase chain reaction was performed for EBV detection.* ***Results****: The study included a total of 28 patient biopsies proven NPC and 32 control individuals. A significantly higher prevalence of EBV in patients as compared with controls. The presence of the EBV infection was determined in half of the NPC patients and 6.3% of the control group. There was none statistically significant association between the presence of EBV and the clinicopathological features.* ***Conclusion:*** *EBV is prevalent in the NPC cases compared with controls. Further studies with a larger sample size are required to determine the true burden of EBV-associated NPC in Egypt*

INTRODUCTION

The Pathogenesis of nasopharyngeal cancer (NPC), a different form of head cancer, is complex and poorly understood, with several risk factors. and unbalanced geographical distribution[1,2] For many years, NPC has been endemic in East and Southeast Asian native populations, Arctic, North African, and Middle Eastern indigenous people for many years[3]. Geographical persistence of this cancer indicates that genetic and/or persistent environmental predisposing factors play a significant role in NPC development. The pathophysiology of NPC is further enhanced by the Epstein-Barr virus's (EBV) involvement [4].

The World Health Organization (WHO) considers three histological types of NPC based on differentiation grade. Keratinizing epidermoid carcinomas can be classified into Type I or the non-keratinizing carcinomas, Type II, or the undifferentiated carcinomas, and lymphoepitheliomas, that is called Type III. All three types are distinguished by a significant lymphocytic infiltration. This interaction between lymphocytes and tumor cells seems very important for the continuous spread of the Type III2 malignant carcinoma components. Of these, variants II and III are the most frequent and have common etiological characteristics, related to the Epstein–Barr virus (EBV) infection [5-7].

Epstein–Barr virus, Previously referred as human herpesvirus 4, that is categorized under the Lymphocrytovirus genus of the Gamma herpesvirinae subfamily within the Herpesviridae family [8]. EBV is categorized in group I carcinogen and is responsible for 1.5% of all human cancer cases and 1.8% of cancer-related fatalities. These cases include gastric carcinoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, Burkitt lymphoma, and B-cell, T-lymphocytes, and NK-cell lymphomas. Eighty-two percent of EBV-linked malignancies and eighty-nine percent of EBV-associated neoplasm-related fatalities were attributed to gastric carcinoma and nasopharyngeal carcinoma (NPC)[9].

NPC occupies a special place among the epithelial neoplasms associated with EBV. The default program for EBV infection in epithelial cells of nasopharynx is lytic infection. Consequently, maintaining latency and changing the mode of infection from lytic to latent are crucial steps in pathogenesis of NPC [10] In endemic regions of NPC, EBV accounts for 95% of NPC incidences and 100% of NPC-related mortalities. In contrast, in low-incidence areas, EBV is accountable for twenty percent of the nasopharyngeal cancer incidences and eighty percent of NPC mortality [11]. This study aims

to investigate the prevalence of Epstein Barr Virus in Patients with nasopharyngeal carcinoma in Egypt and the associations between Epstein Barr Virus infection and clinicopathological and demographic characteristics.

METHODOLOGY

Study group:

The current study comprised random patients diagnosed with a biopsy-proven pharyngeal cancer and hospitalized at the Otolaryngology Division, Mansoura University Hospital in Egypt from September 2021 to August 2023. Patients with occupational exposure to hazardous chemicals or post-chemotherapy/radiotherapy were excluded from the current study. The tumours were staged by the TNM system. The TNM classification was done according to the criteria of the Union Against Cancer (UICC) [12]. Histological grading was performed based on World Health Organization criteria, which divide tumors into three categories: well-differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) [13].

Control group:

Persons with a confirmed diagnosis of non-cancerous pharyngeal lesions admitted for microsurgery nodules, cysts, granulomas, nasal polyps, or adenoid tissue. This group is free from any cancer or any history of previous cancer.

Fresh tissue biopsies were collected during surgery after informed patients' consent. The research was accepted by the Mansoura Faculty of Medicine, ethical committee, Institutional Research Board (IRB), code No. MD.21.07.501.

Molecular testing:

DNA isolation from Fresh Frozen tissue was done using the QIAamp/ DNA/Minikit/ Qiagen, (cat. no. 51304) according to manufacturer instructions. Immediately after the isolation, the purity and efficiency of the eluted samples were checked by a Nanodrop One spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) with a 260/280 nm absorption ratio. Isolated samples were kept at −20˚C.

For viral detection, real-time polymerase chain reaction (RT-PCR) was performed. Primers from *EBNA -1* (213 bp) region was used for EBV detection and along with primer for *beta-globin gene (*286bp) used as a positive control. The primer sequences utilized are listed in **Table 1.**

Table 1: Synthesized primers for DNA, Epstein-Barr virus (EBV), and (Epstein-Barr nuclear antigen-1 *(EBNA-1)*

	Genes	Primers sequence, 5'–3'	Reference
1	***EBNA-1 QP1 Fwd.***	5'-GCCGGTGTGTTCGTATATGG-3'	***Vanshika et al.*** [14]
2	***EBNA-1 QP2 Rev***	5'-CAAAACCTCAGCAAATATATGAG-3'	
3	***Beta-Globin Fwd.***	5'-TAGCAACCTCAAACAGACACCA-3'	
4	***Beta-Globin Rev***	5'-CAGCCTAAGGGTGGGAAAAT-3'	

The reaction mixture consists of 1 microliter of both forward and reverse primer, 2 µl nuclease free water, and 6 µl of eluted sample DNA in PCR tubes with 10 µl of SYBR green The HERA SYBR® Green qPCR kit, Willofort.co.UK). For every sample, the *beta-globin gene's TAL57* region was used as PCR inhibitors control, and quality control for the DNA. Additionally, negative tests were conducted utilizing PCR-grade water. The following were the cycle runs for *beta-globin*, and EBV *EBNA-1 gene* 1) 3 minutes at 95°C; 2) 40 cycles of 95°C for 15 seconds; 3) 60°C for 20 seconds; 4) 72°C for 25 seconds; 5) 7 minutes at 72 °C [14].

Statistical analysis:

Data entry and analysis were done by using IBM-SPSS software (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.). Qualitative data was expressed as N and percentage (%). Quantitative data was initially examined for normality using Shapiro-Wilk's test with data being normally distributed if p>0.050. The presence of significant outliers was tested for by inspecting the boxplots. Quantitative data was expressed as mean and standard deviation. For qualitative data, the test of chi-square was applied depending on the expected cell counts. The chi-square test was used if expected count in all cells is 5 or more, otherwise, Fisher's exact cross tabulation test (for 2 x 2 crosstabulation) or Fisher-Freeman-Halton test (for larger crosstabulation) was used. For quantitative data for two groups, the independent-samples t-test was applied. For every test that is utilized, results were defined as statistically significant if *p-value* $\leq$ 0.050. When required appropriate charts were used to graphically display the results.

RESULTS

The study included 61 patients, including 28 NPC patients with pathological confirmation and 33 control individuals unrelated to any malignancy. The majority of NPC patients include (53.6%) males and (46.4%)

females with mean age 50.57 and age range from (14-76) years. The control group consists of 33 individuals; this group included (43.8%) males and (56.2%) females with age range from (7-50) years. The majority of tumours were type 2 (60.7%). The tumour staging characteristics of NPC were summarized in **Table2.**

Table 2: Tumor characteristics of Nasopharyngeal carcinoma group (n=28)

Characteristic	N	%
Nasopharyngeal WHO type		
Type1	0	0.0
Type 2	17	60.7
Type 3	11	39.3
T stage		
T0	1	3.6
T1	10	35.7
T2	10	35.7
T3	6	21.4
T4	1	3.6
N stage		
N0	1	3.6
N1	14	50.0
N2	9	32.1
N3	4	14.3
M stage		
M0	25	89.3
M1	3	10.7

The EBV presents in 50.0% (14/ 28) of NPC cases with a statistically significant difference higher *(p-value* ≤ .001) than in the control group 6.3% (2 /32). The main NPC and control group characteristics were displayed in **Table 3.** Comorbidities showed a difference between the two groups that is statistically significant *(p-value* ≤ .001).

Table 3: Residence, smoking, and EBV positivity rate between Nasopharyngeal carcinoma patients and control group.

Characteristic	Nasopharyngeal carcinoma		Nasopharyngeal control		Sig.
Categorical	**N=28**	**%**	**N=32**	**%**	
Residence					
Rural	20	71.4	21	65.6	.630
Urban	8	28.6	11	34.4	
Comorbidities	13	46.4	1	3.1	**<.001**
Current smoking	6	21.4	2	6.3	.130
Positive EBV	14	50.0	2	6.3	**<.001**

Notes: SD = standard deviation. Sig. = p-value. The tests of significance are chi-square, Fisher's exact, or Fisher-Freeman-Halton tests for categorical data. This table shows a statistically significant different higher EBV positivity in nasopharyngeal carcinoma vs. control group.

Demographic and clinicopathological characteristics according to the Status of EBV infection in the NPC group were included in **Table 4.** Female sex predominated in positive cases for EBV (64.3%) vs. EBV negative cases with (28.6%) with *P-value* equal to 0.058. However, no significant statistical difference concerning residence, and comorbidity.

Table 4: Demographic and clinicopathological parameter in accordance to EBV PCR positivity.

Characteristic	Positive EBV		Negative EBV		Sig.
	N=14	%	N=14	%	
Male sex	5	35.7	10	71.4	**.058**
Residence					
Rural	11	78.6	9	64.3	.678
Urban	3	21.4	5	35.7	
Comorbidity					
Diabetes	6	42.9	1	7.1	.077
Hypertension	6	42.9	5	35.7	.699
Hepatitis C	2	14.3	1	7.1	1.000
Current smoking	0	0.0	6	42.9	.016
Nasopharyngeal WHO type					
Type 1	0	0.0	0	0.0	
Type 2	10	71.4	7	50.0	.246
Type 3	4	28.6	7	50.0	
T stage					
T0	0	0.0	1	7.1	
T1	7	50.0	3	21.4	.343
T2	4	28.6	6	42.9	
T3	2	14.3	4	28.6	
T4	1	7.1	0	0.0	
N stage					
N0	1	7.1	0	0.0	.095
N1	9	64.3	5	35.7	
N2	4	28.6	5	35.7	
N3	0	0.0	4	28.6	
M stage					
M0	14	100.0	11	78.6	.222
M1	0	0.0	3	21.4	
	Mean	**SD.**	**Mean**	**SD.**	**Sig.**
Age (Years)	50.14	18.761	51.00	13.502	.109

Notes: SD = standard deviation. Sig. = p-value. The tests of significance are chi-square, Fisher's exact, or Fisher-Freeman-Halton tests for categorical data, and independent-samples t-test for numerical data.

DISCUSSION

EBV infection is identified as an early and necessary step in nasopharyngeal tumorigenesis development, played an important role in the disease carcinogenesis and are observed in the majority of nasopharyngeal cancer [15]. EBV-associated NPC showed a skewed geographical distribution despite the fact that 90% of adult humans worldwide are infected with the virus [16].

As regard Nasopharyngeal WHO typing, most of our cases were type II & III only. T1 and T2 were the most prevalent along with N1 stage. In our study, only 10.7 % of NPC patients had cancer metastasis in consistence with other studies [(17)].

In our study, males (53.6%) were more commonly affected by NPC than females which has been observed in a study in Sudan [18] that has been previously reported that the number of males who have NPC was higher than females. Also, This male predisposition to head and neck cancers had been observed in many other studies [19, 20]. This can be clarified by biological or gender differences or different lifestyles in the prevalence of some NPC environmental risk factors

The majority of NPC patients in the current study presented in the fifth decade of life, In agreement with National Cancer Institute [21] that highlighted that head and neck cancers were more commonly diagnosed in individuals over the age of 50 years.

In the current study, EBV was positive in half of the NPC cases , which was consistent with Salano et al. [22] who reported EBV presence in about (45%) of NPC cases, also is similar to the prevalence in Sudan (61.3%) [23]. Interestingly, South African NPC patients had a significantly higher EBV incidence. By using PCR to amplify the EBER region, Janse and his colleagues [24] examined the incidence of EBV in 38 NPC patients, their study indicates that EBV was found in 82 % (31/38) of the tumours. Increased prevalence of infection by EBV was also reported in Nasopharyngeal cancer patients in previous studies, in Saudi Arabia

(96%) [17,] China [24], and Turkey (87%) [25]. On the contrary, a previous report from Ghana Asante et al. [26] showed a low 25% detection rate of EBV in NPC. This discrepancy can be the consequence of DNA degradation in NPC samples embedded in paraffin. Therefore, contrary to the fresh samples used in this study, molecular data from these types of samples (FFPET) is critical

Our study showed that EBV was present in 6.3% of the control samples. this is in agreement with Studies conducted on exfoliated nasopharyngeal cells from healthy individuals in a high-risk population which revealed that 13% of the subjects had EBV, which is likely due to prior EBV exposure [27]. EBV detection in adenoid lesions suggests that further genetic and many environmental factors other than viral presence are necessary for tumour initiation and progression. Also Jiang et al. [28] indicated that EBV can be present in the healthy epithelial cells as latent virus.

As regarding sex distribution with EBV infection in NPC, female sex predominates in EBV positive cases (64.3%) vs. negative cases (28.6%) This finding is consistent with the study done by Asante et al. [26] where they reported a higher female percentage regarding EBV in NPC and with Ayee et al. [29] who found that females being two times more at risk of having EBV infection compared with males. This percentage variation could result due to the group different life style and the viral different routes of entry [26].

In Nasopharyngeal cancer (NPC) cases, the observed results showed insignificant statistical differences between the oncogenic viral status and age that was in agreement with Laantri et al. [30] and Ritchie et al. [31].

In the present work, there was a predominance of WHO type II; nasopharyngeal cancer with EBV genome (71.4%) followed by type III (28.6%) which is in agreement with previous studies which reported that the non-keratinizing types (types II and III), especially the undifferentiated histopathologic type, has a strong association with EBV infection [23]. This study result is in disagreement with findings of Asante et al. [26] that reported EBV DNA was only present in 32.14% of the total number of type III EBV positive cases, None was detected in both types I and II.

The most common stage in EBV positive NPC was T1 and N1 which is inconsistent with Shao et al. [32] as regard N stage results only. There is no significant difference in metastasis between positive and negative EBV NPC patients in agreement with a Six-Year Cross-Sectional Study Al-Anazi et al. [17].

CONCLUSION

Despite the current study shows that about half of NPC have EBV infection; a larger sample size study is required to assess the viral burden in Nasopharyngeal cancers and other head and neck cancer.

Abbreviations:

Epstein Barr Virus (EBV), nasopharyngeal carcinoma (NPC), Union Against Cancer (UICC), Institutional research board (IRB), real-time polymerase chain reaction (RT-PCR), Epstein–Barr nuclear antigen 1 (EBNA1).

Declarations:

Consent for publication: Not applicable

Availability of data and material: Data are available upon request.

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