# Association of Epstein- Barr Virus (EBV) with the Development of Nasopharyngeal Carcinoma (NPC) in Western Region of Iraq: Unmatched Molecular Case-Control Study

**Mothana Ali Khalil[1], Arkan Obaid Jasim Al-Essawi[2], Mohammed Tafash Dagash[3]**

*[1]Assistant Professor /Department of Microbiology/Virology, College of Medicine, University of Anbar, Iraq, [2]Assistant Professor /Department of Pathology, College of Medicine, University of Anbar, Iraq, [3]Assistant Professor /Department of Surgery /Thoracic Surgery, College of Medicine, University of Anbar, Iraq*

## Abstract

**Background:** Nasopharyngeal Carcinoma (NPC) is uncommon in Iraq, but its incidence is raising due to increased exposure to diverse risk factors. Many of the NPC-related risk factors are becoming more and more apparent in Iraq. The exactly risk factors for nasopharyngeal carcinoma (NPC) in Anbar province, Iraq are not known.

**Objectives:** To determine the association between Epstein- Barr virus (EBV) infections and other risk factors with the development NPC of Iraqi patients.

**Patients and Method:** Sixty-seven paraffin-embedded tissues of NPC cases, 134 normal noncancerous nasopharyngeal biopsy samples, and tonsillectomy specimens from patients with chronic hypertrophic tonsillitis as controls were enrolled in the study that was conducted between 12 January 2012 and 21 January 2019. DNA of EBV was extracted from both controls and neoplastic tissues and analyzed by PCR technique using primers specific to EBV Latent Membrane Protein-1 Oncogene (LAMP-1) for the presence of EBV. A Questioners data form for all patients and controls were filled by the researchers regarding other risk factors of NPC including the patient's age, sex, residence, radiation exposure, history of chronic rhinitis, family history of NPC, tobacco smoking, alcohol consumption, herbal medicines, tea consumption, exposure to formaldehyde and exposure to different inhalants.

**Results:** The following risk factors were found to be independently associated with illness: EBV (adjusted odds ratio [OR] 7.852, 95% confidence interval [CI] 2.22–27.72), herbal medicine (OR 19.051, CI 7.56–47.95) and Family history of NPC (OR 63.717, CI 6.67–607.96).

**Conclusion:** Combination of family history of NPC , EBV exposure and herbal medicine was a strong risk factor for NPC.

***Keywords:*** *Epstein - Barr virus, Nasopharyngeal Carcinoma, PCR, EBV-LMP-1, other risk factor, case-control study*

## Introduction

Nasopharyngeal carcinoma (NPC) is a human malignancy derived from the epithelium of the retro-nasal cavity and it is one of the most frequent head and neck cancers with elevated prevalence rates in Asia and western North Africa [1, 2]with, approximately 86691 incident cases of NPC and 50 831 NPC-related deaths in 2012 worldwide [3, 4].

Previous studies demonstrated Epstein –Barr virus (EBV) infection, genetic susceptibility, diet, chromosomal disorders, aberrant promoter hyper methylation and other genetic related factors and other environmental exposures [1, 5, 6], Cigarette smoking, alcohol consumption and Consumption of salted [1, 7, 8], Family history of NPC) **,** formaldehyde, an industrial hygienist solvents, dusts, exhaust and pesticides have been suggested to be associated with increased NPC risk [1, 9]. The present study to know the roles of EBV with

other Risk factor in development of NPC.

## Materials and Method

### Study design

Case-control sets were included for analysis, provided that two control was matched for each case by age, sex and general geographical location of the case's residence. A standard questionnaire for all patients and controls were filled by the researchers regarding other risk factor of NPC. Including the patient's age, sex, residence, Radiation exposure, history of chronic Rhinitis, family history of NPC, tobacco smoking with alcohol consumption, herbal medicines, Tea consumption, Exposed to formaldehyde and, exposure to different inhalants. This study was conducted in the Anbar province and its neighbor area and biopsies were collected over a 7 years period between 3 January 2012 and 1 January 2019. The protocol was approved by the Microbiology Department, Anbar medical College. All patients provided informed consent for participation in the study and for biopsy samples taken from the tumors and tonsillectomy specimens.

### Molecular study of EBV

### Patients and Methods:

Sixty-seven paraffin-embedded tissues of NPC cases and 134 normal noncancerous nasopharyngeal biopsy samples and tonsillectomy specimens from patients with chronic hypertrophic tonsillitis as controls were enrolled in the study that was conducted between 3 January 2012 and 1 January 2019 to investigate the presence of LMP-1 gene. Paraffin-embedded tissues of NPC cases were selected from the archives of private pathology laboratories in Anbar province and cases were classified according to the WHO classification [10]. Archived slides were reviewed by two pathologists for confirmation of diagnosis and tissue adequacy for extracting of DNA. Controls were matched on gender, residence and year of birth. The molecular analyses were carried out at private laboratories in Baghdad city.

### Tissue Processing, PCR Amplification and Gel Electrophoresis

The tissue samples were purified from archival paraffin embedded tissue blocks as described previously [11]. DNA of EBV was extracted from both cases and controls using the same procedure described previously [12] and PCR amplification for detection of EBV-LMP1 gene were processed through using oligonucleotide (sense BN1, antisense BN2) as follow (sense BN1: 58-AGC GAC TCT GCT GGA AAT GAT-38 or antisense BN2: 58-TGA TTA GCT AAG GCA TTC CCA-38) as described previously [13]. The products were then examined on 1.5% agarose gel electrophoresis in 1× Tris-boric acid–EDTA (TBE) solution and stained with ethidium bromide to verify the presence of 316 bp PCR product.

## Statistical Analysis

A matched design and a case-control ratio of 1:2 was selected as the most appropriate strategy in order to maximize the study power. To detect an association with a matched odds ratio of 2.0 at the 5% significance level with 80% power (assuming 20% exposure level among controls), a sample size of 67 cases and 134 controls was required [14].

Epi Info Version 7.02 [15] and SPSS version 24 were used to calculate crude matched odds ratios (OR) with 95% confidence intervals (CI) and two-tailed P-values to estimate the association between various potential risk factors and NPC. Following the univariate analysis, SPSS were used to calculate adjusted odds ratios by conditional binary logistic regression of risk factors with a P-value < or = 0.25.

## Results

During the study period, 67 NPC cases were collected and 134 normal lymphatic tissues registered as possible controls. From the total number of controls registered, 134 were included in the study,67cases matched to 2 controls. The demographic characteristics of the cases and controls are shown in Table 1. More males than females were enrolled in the study, with the majority of subject's in age group 19 – 38 (40.3%) and 58 years old and over (49.3%). Regarding residence, rural patients more than urban.

**Table 1: Demographic characteristics of subjects**

| Character | NPC Cases N= 67 (%) | Healthy Control N= 134 (%) | P. Value |
|---|---|---|---|
| Gender<br>Male<br>Female | 50 (74.63%)<br>17 (25.37 %) | 100 (74.63%)<br>52 ( 25.37 ) | 1.000 |
| Residence<br>Rural<br>Urban | 42(62.69 %)<br>25 (37.31 %) | 84 (62.69 %)<br>50 (37.31 %) | 1.000 |
| **Age Group** | | | |
| <= 18 | 1(1.5%) | 2 (1.5%) | 1.000 |
| 19 - 38 | 27 (40.3%) | 54 (40.3%) | |
| 39 - 57 | 6 (9.0%) | 12 (9.0%) | |
| 58+ | 33 (49.3%) | 66 (49.3%) | |

**Detection of EBV LAMP1**

EBV were Detected in NPC cases and healthy cases on Agarose gel electrophoresis stained with Rad safe as shown on Fig.1

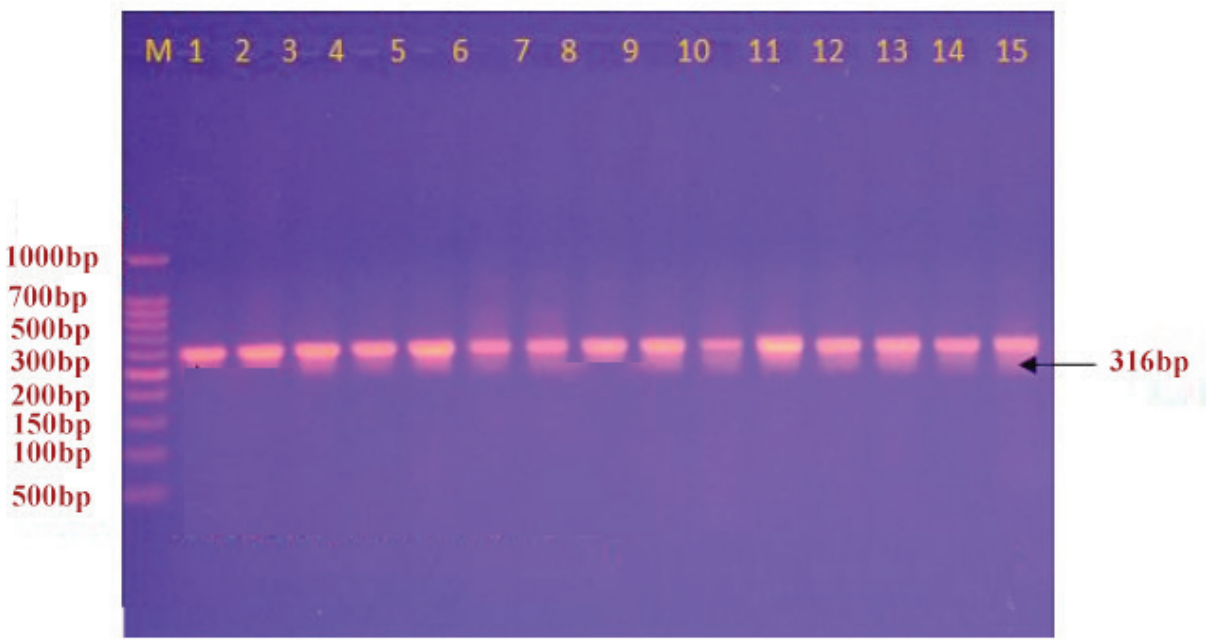


**Fig. 1: Detection of EBV in NPC cases and healthy cases on agarose gel electrophoresis stained with Rad safe. Lane M shows a molecular size marker. Lanes 1-15 shows the positive bands of EBV-wt-LMP1 with product size 316 bp.**

**Univariate analysis of risk factors**

Potential risk factors for NPC (i.e. those with a matched odds ratio > 1) are shown in Table 2. Infection with EBV (OR 10.631, CI 3.654-30.930), Herbal Medicine (OR 20.7955, CI 9.531-45.457), history of chronic Rhinitis (OR 3.792, CI 1.069-13.447), Family history of NPC (OR 74.233, CI 9.752-565.051) and Exposed to formaldehyde (OR 2.375, CI 1.303-4.330), were highly and moderate risky and significantly associated with NPC.

Cigarette Smoking (OR 1.309, CI 0.727-2.356) with a low risk of illness, but this was not statistically significant.

In regard to Alcohol drinking (OR 0.715, CI 0.381-1.342), High Background Radiation Areas (OR 0.662, CI 0.068-6.484), Tea consumption (OR 0.557, CI 0.307-1.010) and Dust and/or exhaust exposure/domestic fumes intake (OR 1.031, CI 0.569-1.870) were non significantly associated with NPC

Insufficient data were obtained on Cannabis intake, Salted fish consumption and history of chronic respiratory tract conditions that may be assumed or potential risk factor for NPC, and as such, no conclusions could be made about these exposures.

**Table 2: Univariate analysis of selected risk factors for NPC (matched OR > 1)**

| Risk Factors | NPC Cases N= 67)) | Healthy Control N= 134)) | Matched Odds Ratio | 95% CI | P. Value |
|---|---|---|---|---|---|
| EBV Positivity | | | | | |
| Positive | 63 (94.03 %) | 80 (59.70 %) | 10.631 | 3.654-30.930 | 0.000 |
| Negative | 4 (5.97 %) | 54 (40.30 %) | | | |
| Herbal Medicine | | | | | |
| Yes | 45 (67.16 %) | 12 (9.00 %) | 20.7955 | 9.531-45.457 | 0.000 |
| No | 22 (32.84 %) | 122 (91.00 %) | | | |
| History of chronic Rhinitis | | | | | |
| Yes | 7 (10.45 %) | 4 (2.99 %) | 3.792 | 1.069-13.447 | 0.044 |
| No | 60 (89.55 %) | 130 (97.01 %) | | | |
| Family history of NPC | | | | | |
| Yes | 24 (35.82 %) | 1 (0.75 %) | 74.233 | 9.752-565.051 | 0.000 |
| No | 43 (64.18 %) | 133(99.25 %) | | | |
| Exposed to formaldehyde | | | | | |
| Yes | 36 (53.73 %) | 44 (32.84 %) | 2.375 | 1.303-4.330 | 0.006 |
| No | 31 (46.27 %) | 90 (67.16 %) | | | |
| Cigarette Smoking | | | | | |
| Smoker | 35 (52.24 %) | 61 (45.52 %) | 1.309 | 0.727-2.356 | 0.347 |
| Nonsmoker | 32 (47.76 %) | 73 (54.48 %) | | | |
| Alcohol drinking | | | | | |
| Yes | 20 (29.85 %) | 50 (37.31 %) | 0.715 | 0.381-1.342 | 0.347 |
| No | 47 (70.15 %) | 84(62.69 %) | | | |
| High Background Radiation Areas | | | | | |
| Yes | 1 (1.49 %) | 3 (2.24 %) | 0.662 | 0.068-6.484 | 1.000 |
| No | 66 (98.51 %) | 131(97.76 %) | | | |
| Tea consumption | | | | | |
| Yes | 34 (50.75 %) | 47(35.07 %) | 0.557 | 0.307-1.010 | 0.067 |
| No | 33(49.25 %) | 87(64.93 %) | | | |
| Dust and/or exhaust exposure/domestic fumes intake | | | | | |
| Yes | 28 (41.79 %) | 55 (41.04 %) | 1.031 | 0.569-1.870 | 1.000 |
| No | 39 (58.21 %) | 79 (58.96 %) | | | |

**Multivariate analysis of risk factors**

Of the ten risk factors included in the conditional binary logistic regression model, a significant independent association with illness was found for the following three risk factors: exposure to EBV (adjusted OR 7.852), Herbal Medicine using (adjusted OR 19.051), and Family History NPC (adjusted OR 63.717) (Table 3).

**Table 3: Multivariate analysis of selected risk factors for NPC**

| Risk factor | Adjusted odds ratio | 95% CI | P-value |
|---|---|---|---|
| EBV Positivity | 7.852 | 2.224-27.720 | 0.001 |
| Herbal Medicine using | 19.051 | 7.568-47.953 | 000 |
| Family History NPC | 63.717 | 6.678-607.963 | 000 |

## Discussion

The results obtained from our study suggest a strong effect of exposure to EBV, herbal medicine using and family history of NPC on NPC risk, Exposure to formaldehyde and the history of chronic rhinitis were also related to NPC risk, whereas the exposure to high background radiation, cigarette smoking, alcohol drinking, tea consumption and dust and/or exhaust exposure/domestic fumes intake in Anbar province were not related to NPC risk even after adjusting for major risk factors of NPC. Epidemiological study have combined three suspected risk factors (EBV, herbal medicine using and Family History NPC) into a simultaneous analysis factors and study their possible synergetic effects, and reports of relatives with NPC using conditional multivariate logistic regression.

Therefore, it is strongly suspected that NPC risk is affected by cofactor(s) in addition to EBV infection like herbal medicine using and family history NPC were strong risk factors of NPC. This finding is consistent with that of Sriamporn et al.(1992) [16] who showed elevated VCA/IgA and neutralizing antibodies against EBV DNAse with a 20-fold increase in NPC risk for subjects seropositive for VCA/IgA antibodies, and a 30-fold increase for those seropositive for both biomarkers.

Our study agreed with two studies from Taiwan that showed high prevalence of the LMP1. DNA (94.7–100%) in swab samples from NPC patients but not from control groups and other study that demonstrated that NPC is well specimen that can be demonstrate the transforming ability of EBV latent membrane protein which is expressed in approximately 65% of NPC tumors [5, 18, 19], So the plasma EBV-DNA level might be a sensitive and reliable biomarker for the diagnosis of NPC at a molecular level/ clinical practice [20, 21] as EBV is a major etiologic factor for (NPC), and it is detected in tumor cells of virtually all NPC cases [22].

The results from Tables 2 suggest a synergetic effect between herbal medicine using and EBV. These results are in agreement with those obtained by West et al [23] that confirms a 2- to 4-fold excess risk of NPC in association with use of traditional herbal medicines through several case control studies. In contrast to other findings, however, no evidence of herbal medicine using links to NPC was detected [1]. Herbal medicine using might be a strong risk factor of NPC through activation EBV in latent EBV infected cell or through a direct promoting effect on EBV-transformed cells [24] .

Furthermore Family History NPC (indicated as a history of first degree relatives with NPC) was risky for NPC as approved by Busson et al, (2004) [5] who found there is evident that genetic factors might be of importance for the etiology of NPC. The mechanism of familial clustering is not understood and it may reflect genetic factors, shared environmental factors, or both [9]. In accordance with the present results, previous studies have demonstrated that a strong association between our estimates of formaldehydeexposure and NPC [9].

The present study identifies cigarette smoking, Alcohol drinking, Tea consumption and dust and/or exhaust exposure/domestic fumes intake are statistically non-significant risk factors for NPC after adjusting these identified risk factors. These findings are in contrary to some studies that suggested these factors are risk factor for NPC. However, other studies which have suggested

that the relationship of NPC to these risk factors are less clear and inconsistent [1, 7-9].

To our knowledge, this study is the first study in Anbar province that illustrates the association between some risk factors including EBV and NPC development, hoping that a better understanding of the etiologic interactions between viral and environmental factors in the pathogenesis of NPC.

## Conclusion

Combination of family history of NPC, EBV exposure and herbal medicine was a strong risk factor for NPC.

**Ethical Clearance**: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

**Conflict of Interest**

The authors declare that they have no conflict of interest.

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