

# Molecular Detection of Epstein Barr Virus (EBV) Among Sudanese Patients with Esophageal Cancer

## Abstract

**Background:** Esophageal cancer remains public health concern for many countries. The objective of the current study was to identify (if any) Epstein Barr Virus (EBV) in tissue specimens obtained from Sudanese patients diagnosed with esophageal cancer.

**Methodology:** In the current study, we retrieved 102 formalin fixed paraffin wax processed tissue's blocks obtained from patients with esophageal carcinomas. All tissue samples were retrieved from histopathology laboratories in Khartoum City, Sudan and subsequently investigated by PCR method for the presence of EBV. All specimens were previously diagnosed with esophageal carcinoma.

**Results:** Out the 102 patients, 6 (5.88%) were found to be positive for EBV by PCR, while 96 (94.12%) were found to be negative. Of the 56 males 5/56 (8.9%) were found to be positive for EBV and 51/55(91.1%) were found to be negative. Of the 46 females, 1/46 (2.2%) were found to be positive for EBV and 45/46(97.8%) were found to be negative. The risk associated with male sex was and OR (CI) = 4.5(0.51-39.99), P = 0.18.

**Conclusion:** EBV might play some roles in the etiology of esophageal carcinoma in Sudan. Sustainable preventive strategies are required to reduce the epidemiology of esophageal cancer in Sudan.

**Keywords:** Esophageal cancer; EBV; Sudan; Molecular detection; Esophageal carcinoma; Etiology; Adenocarcinomas

## Research Article

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**Mohmad Ahmad Babikir Ibraheem Beely<sup>1,2</sup>, Hussain Gadelkarim Ahmed<sup>3,4\*</sup>, Mohammed Siddig Abd El Aziz<sup>2</sup>, Ahmed Abdalla Agab Eldour<sup>1</sup>, Bassam Ahmed ALmutlaq<sup>3</sup>, Abdulrahman Ahmed Almalaq<sup>3</sup>, Ahmed Dirweesh<sup>5</sup> and Fahd Z Eissa<sup>3</sup>**

<sup>1</sup>Department of Medical Laboratory, Faculty of Medicine, University of Kordofan, Sudan

<sup>2</sup>Department of Histopathology and Cytology, Faculty of Medical Laboratory Science, Sudan University for Science and Technology, Sudan

<sup>3</sup>Department of Pathology, College of Medicine, University of Hail, KSA

<sup>4</sup>Department of Histopathology and Cytology, FMLS, University of Khartoum, Sudan

<sup>5</sup>Seton Hall University School of Health and Medical Sciences, St Francis Medical Center, USA

**\*Corresponding author:** Hussain Gadelkarim Ahmed, Department of Pathology, College of Medicine, 2440, University of Hail, Kingdom of Saudi Arabia, Email: hussaingad1972@yahoo.com

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**Abbreviations:** EBV: Epstein Barr Virus; EC: Esophageal Carcinoma; AC: Adenocarcinomas; CMV: Cytomegalovirus; LMP1: Latent Membrane Protein 1; SDS: Sodium Dodecyl Sulfate; EBNA-4: EBV Nuclear Antigen-4; RRs: Relative Risks; NPC: Nasopharyngeal Carcinoma; PCR: Polymerase Chain Reaction

## Introduction

Esophageal carcinoma (EC) is one of the most frequent cancers, particularly in Asia, and it is the sixth cause of the global cancer deaths [1]. Most of the esophageal carcinomas developed from the stratified squamous epithelial lining of the esophagus, hence, adenocarcinomas (AC) arising from columnar glandular cells that replace the squamous epithelium cells [2].

Several risk factors have been incriminated for the development of esophageal cancer such as; viruses [3], tobacco smoking, alcohol drinking [4], gastro-esophageal reflux [5], susceptibility genes [6], low levels of intake of fruits and vegetables [7]. The association between viral infection and esophageal cancer was well established for several viruses including; human papillomavirus, particularly subtype 16, herpes simplex virus 1, Epstein-Barr virus (EBV) and cytomegalovirus (CMV). Nevertheless, the exact pathogenesis of the viral action in addition to geographic

variations in infection rates of these viruses yet requires more work [8]. EBV is one of the oncogenic viruses that have been found to be linked to Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, gastric carcinoma, breast cancer, and leiomyosarcoma [9]. However, whether EBV is associated to esophageal SCC still controversial: there are limited studies and their conclusions vary [10]. The association between EBV and several cancers have been reported in many studies from Sudan. Most of these studies inspected the relationship between EBV and cancers such as, Burkitt's lymphoma [11], nasopharyngeal carcinoma [12] and breast cancer [13]. Therefore, the aim of this present study was to identify (if any) EBV in Tissue specimens obtained from Sudanese patients diagnosed with esophageal cancer.

## Materials and Methods

This is a retrospective descriptive study examined 102 Sudanese patients with esophageal cancer of whom, 56 were males and 46 were females (males'/females' ratio, 1.20:1.00), their ages ranged between 21 and 98 years with mean age and STD of 59.5± 1.6 years old. These patients with esophageal carcinomas, were investigated for the presence of EBV using LMP1 (Latent membrane protein 1).

## DNA extraction

DNA was extracted adopting procedure described by Ahmed et al. [13]. DNA was extracted from paraffin-embedded samples, by immersing tissue section in xylene to dissolve the paraffin from the tissue, and then rehydrated using a series of ethanol washes. Proteins and harmful enzymes such as nucleases were digested by proteinase K. Buffer containing denaturing agent (sodium dodecyl sulfate (SDS)), was added to facilitate digestion [14]. Nucleic acids were purified from the tissue lysate using buffer saturated phenol and high speed centrifugation. Following phenol extractions, RNase A was added to eliminate contaminating RNA. Additional phenol extractions following incubation with RNase A were used to remove any remaining enzyme. Sodium acetate and isopropanol were added to precipitate DNA, and high speed centrifugation was used to pellet the DNA and facilitate isopropanol removal. Washing with 70% ethanol was performed to remove excess salts, followed by centrifugation to re-pellet the DNA [15,16]. DNA is re-suspended in distilled water; quantified and stored at  $-20^{\circ}\text{C}$ . Purified DNA was subsequently used in downstream applications of PCR.

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## DNA quantification

To evaluate the DNA quantification after DNA extraction, we had analyzed DNA measurement using a NanoDrop spectrophotometer.

## PCR

EBV genome was detected by PCR using two primers that targets EBV nuclear antigen-4 (EBNA-4) 5'-GAGGAGGAAGACAAGAGTGG and 5'-GATTCAGGCGTGGTCCTTGG3' and latent membrane protein-1 (LMP1) 5'-CCGAAGAGGTTGAAAACAAA3' and 5'-GTGGGGTCTGCATCATCTC 3'. Any case detected EBNA-4 and/or LMP-1 was considered positive indicating the presence of EBV.

## Molecular identification by polymerase chain reaction

Polymerase chain reaction (PCR) was carried out for amplification of target EBV genome by using genomic DNA template (1.5 L of EBV detection). PCR was performed in a total volume of 23 L for EBV. Master mix that contained 1 L of 10 mM dNTP mixed (2.5 mM dATP, 2.5 mM dGTP, 2.5 mM dCTP, and 2.5 mM dTTP), 1.5 L of 25 mM  $\text{MgCl}_2$ , 2.5 L of 10 PCR buffer (10 mM TrisHCl [pH 8.3], 50 mM KCl), 1 L Taq polymerase (approximately 1 U), 1.5 L forward primer and 1.5 L reverse primer. Volume was completed to 25 L per reaction mixed with doubled distilled water (ddH<sub>2</sub>O). The master mix of all samples was mixed by vortexing in a sterile 0.2-mL PCR tube. Amplification was then set in an initial denaturation stage at  $94^{\circ}\text{C}$  for 5 minutes, then 30 cycles of  $94^{\circ}\text{C}$  for 30 seconds, and annealing at  $55^{\circ}\text{C}$  for 30 seconds, followed by extension at  $72^{\circ}\text{C}$  for 90 seconds and a final extension stage at  $72^{\circ}\text{C}$  for 5 minutes.

## Ethical Consent

The study was approved by Faculty Research Board, Faculty of Medical Laboratory Science, Sudan University for Science and Technology. This in addition to the fact that, the authors followed the tenants of the Declaration of Helsinki.

## Data Analysis

The data were analyzed for presence of EBV in esophageal cancer tissues. Relative risks (RRs) and 95% confidence intervals (95% CIs) were calculated. Data management was done using Statistical Package for Social Sciences (SPSS version 16). SPSS was used for analysis and to perform Pearson Chi-square test for statistical significance (P value). The 95% confidence level and confidence intervals were used.

## Results

In the current study, we retrieved 102 formalin fixed paraffin wax processed tissue's blocks obtained from patients with esophageal carcinomas of which, 91/102(89.2%) specimens were with squamous cell carcinoma and 11/102(10.8%) were with adenocarcinoma). Of the 102 study subjects 56/102(55%) were males and 46/102(45%) were females their age ranging from 21 to 98 years old with a mean age of 60 years. The majority of the study populations were at the age older than 66 years constituting 38 patients followed by age ranges, (both 56-65 and < 45 years) and 46-55 years, representing 24 patients for each and 16 patients, respectively, as indicated in Figure 1.

Of the 102 patients, 6/102(5.88%) were found to be positive for EBV by PCR, while 96 (94.12%) were found to be negative. Of the 56 males 5/56 (8.9%) were found to be positive for EBV and 51/55(91.1%) were found to be negative. Of the 46 females, 1/46 (2.2%) were found to be positive for EBV and 45/46(97.8%) were found to be negative. The risk associated with male sex was and OR (CI)= 4.5(0.51-39.99), P = 0.18.

Of the 91 Squamous cell carcinomas, 5/91(5.5%) were found to be positive for EBV and 86/91(94.5%) were found to be negative. Of the 11 Adenocarcinomas, 1/11(9.09%) was found to be positive for EBV and 10/11(90.91%) were found negative. The risk associated with Squamous cell carcinoma was, OR (CI) = 0.58(0.06-5.48), P = 0.64. As indicated in Table 1 & Figure 2.

In regard to the association between EBV infection status and age distribution, the highest positive cases were found among elder age ranges 66+ years, representing 4/6(66.7%) followed by 56-65, 46-55, constituting 1/6(16.7%), for each group, whereas, none of the patients at age group < 45 years was found with positive infection, as indicated in Table 2 & Figure 3.

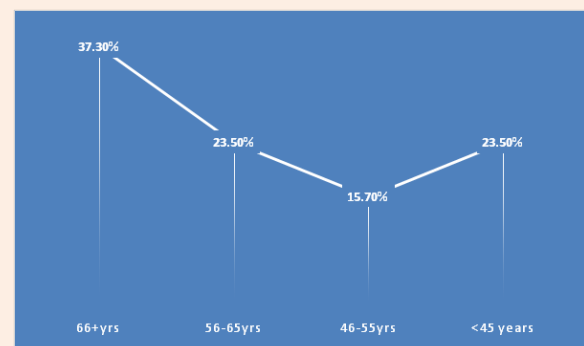


Figure 1: Description of the study population by age.

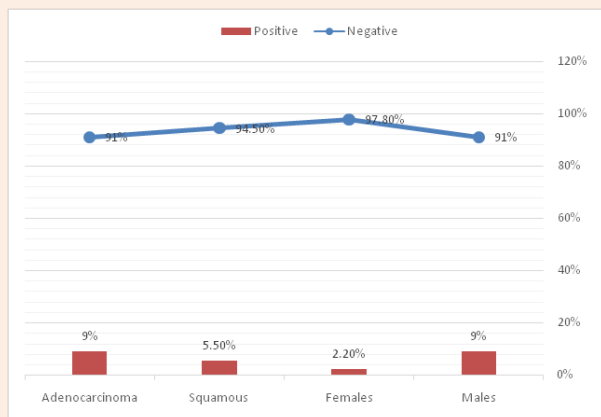


Figure 2: Descriptions of EBV positive with PCR among sex and cancer types.

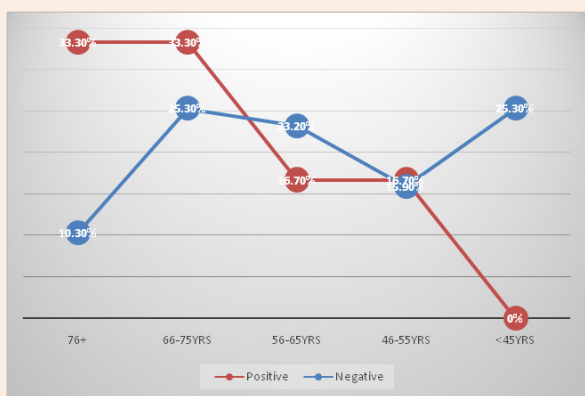


Figure 3: Description of PCR results of EBV by age.

Table 1: Distribution of EBV positive with PCR among sex and cancer types.

Variables	Positive	Negative	Total
Males	5	50	55
Females	1	45	46
Total	6	95	101
Cancer type			
Squamous carcinoma	5	85	90
Adenocarcinoma	1	10	11
Total	6	95	101

Table 2: Distribution of PCR results of EBV by age.

Age group	Positive	Negative	Total
<45 years	0	24	24
46-55	1	15	16
56-65	1	23	24
66+	4	34	38
Total	6	95	102

## Discussion

The etiology of esophageal cancer still obscure; several risk factors have been incriminated which differ for different for geographic regions. Infection with EBV has been proposed as a risk factor for esophageal cancer. Few studies has been performed to clarify the association between EBV and esophageal carcinoma, and the findings are somewhat debated in most occasions [17].

Geographical variations in the incidence of esophageal cancer indicates the variable presence of carcinogens including virtues. In the presence study the prevalence of EBV in the studied samples was approximately 5.9%. To the best of our knowledge no study from the Sudan investigated the link between EBV and Esophageal cancer. Even so, there are some studies from Sudan, which has indicated the presence of EBV in Sudan and linked EBV infection to several cancers. In a study screened Sudanese patients with Nasopharyngeal Carcinoma (NPC), for the presence of Epstein Barr Virus (EBV), using Polymerase Chain Reaction (PCR), and EBV genes were identified in 61.3% of the patients, of whom 63% were found among males and 37% were among females. The study concluded that EBV is prevalent in the Sudan and responsible of the great majority of cases of NPC [13]. In study from Sudan to investigate the association of EBV in childhood leukemia, EBV infections were found in 36.3% of the 80 patients with leukemia [18]. In another study, EBV genome was identified in 55.5% (n=90) of breast cancer tissues as compared to 23% in control tissue samples (p=0.0001). Using ISH, EBV signal was noticed in all 18 breast cancer biopsies inspected whereas, all 5 normal breast tissue biopsies confirmed were negative for EBV. The study present evidence of a strong link between EBV and breast carcinoma in Sudanese patients, and substantial epigenetic silencing of tumor suppressors that may probable be a consequence or an association with viral oncogenesis [19]. Another study investigated 82 tissue samples for the presence of the virus using PCR, EBV was identified in 62.2 % samples and couldn't be identified in 37.8 % tissue samples. Out of the 51 infected tissues, 64.7 % were found among males and 66.7% were found among females [20].

In the present study, EBV infection was identified in 83.3% of the males and only 16.7% of the females. Studies in this context, however, showed conflicting findings in cancers other than esophagus. Nasopharyngeal carcinoma is relatively comparable with our findings [13], whereas, EBV infection is more frequent among females compared to males

In the current study, EBV infection was detected in 83.3% of the squamous cell carcinoma and only 16.7% of the adenocarcinoma. It was found that EBV infection is usually associated with esophageal squamous cell carcinoma rather than adenocarcinoma. In study investigated 72 esophageal squamous cell carcinomas and 40 adenocarcinomas, EBV was detected in 7 cases of the squamous cell carcinoma and in only one case of the adenocarcinoma [21]. Some studies have shown that EBV is associated with carcinoma infiltrating lymphocytes rather than squamous cancerous cells. In a study screened 36 surgically resected esophageal cancer lesions from 36 patients mainly with esophageal squamous cell carcinomas, no EBV positive cancer cell in any tested esophageal squamous cell carcinoma. Numerous EBV positive tumor-infiltrating lymphocytes in the basaloid squamous cell carcinomas [22].

In regard to the association between EBV infection status and age, the highest positive cases were found among elder age ranges 66+ years, representing 66.7%. The relationship between age and EBV infection in esophageal cancer might be attributed to the early exposure to the virus which may somehow be the same as in Burkitt's lymphoma and its link to factors augmenting EBV infection [23]. The penetrating growing of Milk Bush (*Euphorbia*) predominantly in the western parts of Sudan, which accelerate chance of infection with EBV [13].

### Conclusion

EBV might play some roles in the etiology of esophageal carcinoma in Sudan. Sustainable preventive strategies are required to reduce the epidemiology of esophageal cancer in Sudan.

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