DOI: 10.14260/jemds/2015/2047

ORIGINAL ARTICLE

ASSOCIATION OF EPSTEIN BARR VIRUS WITH MUCOSAL SQUAMOUS CELL CANCERS OF HEAD AND NECK

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HOW TO CITE THIS ARTICLE:
Saquib Zaffar, Fir Afroz, Aamir Rashid, Maqbool Lone, Mohammad Ashraf, Mushtaq Sidiqui, Malik Tariq, Arshad Manzoor. "Association of Epstein Barr Virus with Mucosal Squamous Cell Cancers of Head and Neck". Journal of Evolution of Medical and Dental Sciences 2015; Vol. 4, Issue 82, October 12; Page: 14392-14400, DOI: 10.14260/jemds/2015/2047

ABSTRACT: Head and neck cancer is the commonest cancer in India and consists of about one-third of all cancers. Among viral infections, Epstein Barr virus (EBV) has been implicated in the association of many head and neck cancers. Role of EBV as etiological factor in our population remains unknown. **AIMS & OBJECTIVES:** To determine the EBV related etiology of mucosal Head and Neck Squamous Cell Carcinoma (HNSCC) in Kashmir Valley and if positive to define sub-typing of EBV and to find a correlation between the burden of EBV and disease status. **MATERIAL METHODS:** Observational single centre retrospective –prospective study. The study included 53 tissue samples from patients with Squamous Cell carcinoma of Head and Neck region. An equal number of blood samples were taken from healthy volunteers as a control for the reaction process. Sample collection and storage was done as per standard protocol. DNA amplification was done by Polymerase chain reaction. **RESULTS:** We did not observe any sample which tested positive for EBV. All samples were negative for EBV DNA. **CONCLUSION:** EBV has no role in the pathogenesis of Head and Neck Cancers in Kashmiri ethnic population of India. Different Genetic makeup of our population may be responsible for it. Further studies are needed to elucidate the etiology of head and neck Carcinoma in our population.
KEYWORDS: EBV, Head and Neck Carcinoma, Kashmiri population.

INTRODUCTION: Head and neck cancer refers to a group of biologically similar cancers that start in the lip, oral cavity , nasal cavity , paranasal sinuses, nasopharynx, pharynx, and larynx.(1) 90% of head and neck cancers are squamous cell carcinomas (SCCHN), originating from the epithelium of these regions.(2) These cancers are frequently aggressive in their biologic behavior. Patients with these types of cancer are at a higher risk of developing another cancer in the head and neck area.(3) Head and neck cancer is the commonest cancer in India and consists of about one-third of all cancers.(4,5) According to the Indian Council of Medical Research (ICMR) approximately 0.2-0.25 million new head and neck cancer patients are diagnosed each year.(6) In India, head and neck cancers account for 23% of all cancers in males and 6% in females.(7) In Kashmir, Head and Neck Cancer comprises nearly 5% of all malignancies.(8) In males it accounts for 6.4% of all cancers and 3.5% in females.(9) Larynx is the most commonly involved site accounting for 29.4% of all cases.(10)

Risk factors for head and neck cancer include tobacco and alcohol use, ultraviolet (UV) light exposure, viral infection, and environmental exposures.

Among viral infections, Epstein Barr virus (EBV) has been implicated in the association of many head and neck cancers.(11) EBV is a Group I double stranded DNA virus belonging to the family herpesviridae with subfamily gammaherpesvirinae , genus lymphocryptovirus and species human herpes virus 4 (HHV-4).(12) The most common malignancies caused by this virus are Burkitt's lymphoma and Nasopharyngeal carcinoma.(13) Besides nasopharyngeal cancer, it has also been

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detected in other epithelial cancers of the head and neck region including carcinoma of the palatine tonsil, supraglottic laryngeal carcinoma, salivary gland cancer, oral squamous cell carcinoma.[14] EBV has the ability to affect proliferation and survival of its infected host cell that likely renders it oncogenic. The identification of viral DNA and of viral gene products and the immune responses to them now constitute much of the persuasive evidence linking EBV causally to its associated cancers. The saliva of EBV seropositive people is frequently positive for EBV DNA in head and neck cancers. Detection of EBV genome in pathology samples is relevant since its high prevalence in some cancers makes the virus a promising target of specific therapies. Although EBER-RNA in-situ hybridization (RISH) is the standard for EBV diagnosis in tumour cells, the simplicity of polymerase chain reaction (PCR) procedure favours its use as first line method for diagnosis of EBV. Moreover, it has high sensitivity and specificity and helps in subtyping the EBV genome into EBV-1 and EBV-2 strains.

There are conflicting reports with some studies showing strong association of EBV and head and neck Carcinoma while as others show a weak or no association at all. Whether EBV is implicated in the causation of head and neck Carcinoma in our population is unknown. Does EBV remains an etiological factor in our population or does the role of EBV in these malignancies depend on the genetic makeup of different populations remains unknown. This study was designed explore the role of EBV in causation or association with different head and neck Carcinoma.

The present study was undertaken with this background, to ascertain the relationship between EBV DNA/Specific genes in surgical specimen of various Head and neck cancers in the ethnic Kashmiri population of India. This study might give an insight into etiology and hence pathogenesis of these lethal malignancies and help in exploring newer treatment options in future.

AIMS AND OBJECTIVES: To determine the EBV related etiology of mucosal Head and Neck Squamous Cell Carcinoma (HNSCC) in Kashmir Valley and if positive to define sub-typing of EBV and to find a correlation between the burden of EBV and disease status.

MATERIAL METHODS: This study was conducted in Regional cancer centre at Sher-i-Kashmir Institute of Medical Sciences, Soura, Srinagar, J&K, India in collaboration with Department of Immunology and Molecular Medicine. The study included patients with Head and Neck Squamous Cell Carcinoma (HNSCC) registered with Regional Cancer Centre from 2009 to 2012. It was a prospective and retrospective study. Patients having histopathological (HPE) confirmation of the disease were enrolled for the study. An informed consent was taken from all the patients before retrieving the samples for EBV detection. All the clinical details of the recruited patients were studied thoroughly including history, physical examination and investigations. The surgical specimens were retrieved from various Pathology Clinics including Department of Pathology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar, J&K, India. The study included 53 tissue samples from patients with Squamous Cell carcinoma of Head and Neck region. An equal number of blood samples were taken from healthy volunteers as a control for the reaction process.

For detection of EBV through polymerase chain reaction, high molecular weight DNA was isolated from fifty three surgical biopsy samples as well as paraffin embedded tissues from patients with Squamous Cell carcinoma of Head and Neck region. Equal number of peripheral blood samples from age and sex matched healthy subjects were collected and served as a control. Both the test and control samples were stored at -80^{o}C until further processing.

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High molecular weight DNA was isolated from Paraffin embedded tissue or 5-10mg of biopsy sample or 5ml to 10ml of peripheral blood using modified ammonium acetate and phenol - chloroform method. The concentration and purity of the DNA was measured in a spectrophotometer at 260nm wavelength and A_{260} / A_{280} ratio respectively. The integrity of the isolated DNA was analyzed by running 1% agarose gel electrophoresis. The high-molecular-weight DNA thus obtained was amplified by PCR according to the standard procedure**.** The EBV DNA portion in the samples was amplified by using specific primers as reported previously by Decker et al., 1996.[15]

Forward Primer-EBV F 5'-CTTTAGAGGCGAATGGGCGCCA-3' and Reverse Primer-EBV R5'-TCCAGGGCCTTCACTTCGGTCT-3'. A positive EBV DNA was used as an internal control. The standardized non gradient program for the amplification of the target region included 40 cycles of denaturing of DNA at 95^{o}C for 5minutes, annealing at 57^{0}C for 40 seconds, extension at 72^{o}C for 40 seconds and final elongation at 72^{o}C for 7 minutes. The primers amplified the sequence between 14068 and 14562 of the Bam HI-W repeat of EBV to give an amplification product of 495bp which was visualized under UV transilluminator.

OBSERVATION AND RESULTS: A total of 53 patients of Head and Neck Cancer were enrolled in this study for the detection of EBV in their biopsy samples. Biopsy samples of the primary site from patients with Head and Neck Cancer were collected and analyzed for the presence of EBV. The number of male patients was 39 and number of female patients was 14. The mean age was 55.3 years. The distribution as per primary site and subsite of disease is shown in Table 1 and 2 respectively .The distribution as per stage of disease is shown in Table 3. The sex wise distribution of cases as per Stage of disease is shown in figure 1. All the patients had a histology of Squamous Cell Carcinoma from the primary site. The number of patients treated with surgery alone was 7 cases, with radiotherapy alone was 7 cases, with both radiotherapy and chemotherapy was 13 cases, with surgery followed by adjuvant chemotherapy and radiotherapy was 22 cases. Chemotherapy alone was not given in any patient and 4 patients did not receive any treatment at all. The number of fresh tissue specimen was 2 samples and the remaining were wax block samples.

The number of samples in which EBV was detected was none. The distribution of cases according to EBV status is shown in figure 2. The gel picture of EBV gene target is shown in picture 2 which shows amplified DNA from six different samples, all of whom lacked any gene product.

DISCUSSION: "Head and neck cancer" is a collective term defined on anatomical-topographical basis to describe the malignant tumours of the upper aero-digestive tract. About 40% of head and neck cancers occur in the oral cavity, 15% in the pharynx, 25% in the larynx, and the rest in the remaining sites (Salivary glands, thyroid).[16] The causes of head-and-neck cancer are not sufficiently understood. Epidemiological evidence, however, has suggested that many different factors are not necessarily causal agents but are associated, individually or in combination, with an increased probability or risk of the occurrence of these cancers. These are widely denoted as risk factors and are defined as an aspect of personal behavior or lifestyle, or environmental exposure that is directly part of the causal chain or "natural history" of the cancers in question. Of course, all these factors are heavily confounding with socio-economic, cultural and geographic variables and genetic make-up of the ethnic groups.

The present study is the first study designed to study the association of EBV and Head and neck Carcinoma in ethnic Kashmiri population of India.

In humans, EBV is believed to initiate infection in epithelial cells of the oropharynx, which is susceptible to viral replication. Subsequently, lymphocytes migrating through the throat are infected.

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These B-cells are thought to be the source of continued virus spread. Following EBV-latent infection and immortalization of B cells, EBV induces RNA synthesis, immune-globulin secretion, and the expression of B-cell markers, DNA synthesis, and cell division. Several oncogenic gene products that are capable of malignant tumor formation are induced by EBV-latent infection (bcl-2, bcl-10, c-fgr, jun/fos), and certain EBV nuclear antigens have been shown to be oncogenic in their own right (EBNA1, EBNA2, EBNA3A, EBNA3C, LMP1).[17,18,19] These latent proteins are essential for transformation, i.e., immortalization of cell lines.[20]

Among Head And Neck Cancers, the association EBV has been most consistently found with Nasopharyngeal Carcinoma (NPC) while as the association with other sites is quite conflicting in the literature. Although EBV in undifferentiated NPC is non-race related and universal, Chinese patients show a genetic susceptibility to the disease [21].The marker for genetic susceptibility to NPC among Chinese people is the human leukocyte antigen HLA-A, B, and DR locus situated on the short arm of Chromosome 6.In Chinese populations, NPC is associated with certain HLA alleles: A2, Bw46, and B17.Bw46 is also known as Singapore-2 (Sin 2)and is extremely uncommon among Caucasians and other populations.[21] It is clear that a genetic predisposition for NPC exists in certain subgroups, but it is not universal. In our study, we had four patients of Nasopharyngeal Carcinoma, all of which were Type 1 NPC. EBV genome was not detected in any of the samples indicating that EBV may not have a major role in the pathogenesis of NPC in this population. Similar results were obtained in a number of studies done on patients of NPC especially from non-endemic regions.[22-27]

Sino-nasal carcinomas are neoplasms arising in the nasal cavity and paranasal sinuses.[28] Sinonasal carcinomas are strongly associated with EBV in Chinese patients but not in western patients.[29] This suggests that ethnic or geographic influences may be important in determining whether EBV is associated with a given tumor type. In our study, we had 6 cases of sinonasal cancers, all of which were negative for the presence of EBV. This suggests that EBV does not play an important role in development of sino-nasal cancers in our population. Similar results have been observed by various authors in these cancers.[30,31,32]

The possible involvement of oncogenic viruses in oral and oropharyngeal cancers has become a field of increasing interest. EBV could play a role in carcinogenesis, because it is able to infect epithelial cells and its carcinogenicity has been shown.[33] A number of studies have been done to establish the association of EBV with oral and oropharyngeal cancers, the results of which have been conflicting. In our study, we had 28 cases of oral and oropharyngeal cancers in total. EBV was detected in none of the biopsy samples from these sites. Insignificant association of EBV with these cancers has also been seen by various authors.[34,35,36] This suggests that EBV does not have a major role in the pathogenesis of these cancers in our population too.

Laryngeal cancers include supraglottic, glottic and subglottic cancers. Association of EBV with Head and Neck Cancers has been reported quite variably in literature. In our study, we had 15 cases of laryngeal cancer. None of the biopsy samples of these patients was positive for EBV detection. The studies done by various authors have also found similar results,[37,38] this shows that EBV has an unlikely role in the pathogenesis of laryngeal cancers in our population.

Thus in our study on 53 patients of Head and Neck Cancers, EBV was not detected in any of the biopsy samples. This suggests that EBV may not have an etiological role in the pathogenesis of these cancers in our population. This has also been observed by a number of authors in their studies on large cohort of patients.[39,40,41,42]

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CONCLUSION: EBV does not play a significant role in the pathogenesis of Head and Neck Cancers in Kashmir valley. The viral etiology of other carcinomas like HPV in esophageal cancers has also not been found in ethnic population of Kashmir. This signifies that viral infections are not an important etiological factor in the carcinogenesis of malignancies in our population. This may possibly be due to the genetic makeup of our population which renders them less susceptible to oncogenic effect of viral infections. However, further studies are required to elucidate exact etiology of these cancers in our population.

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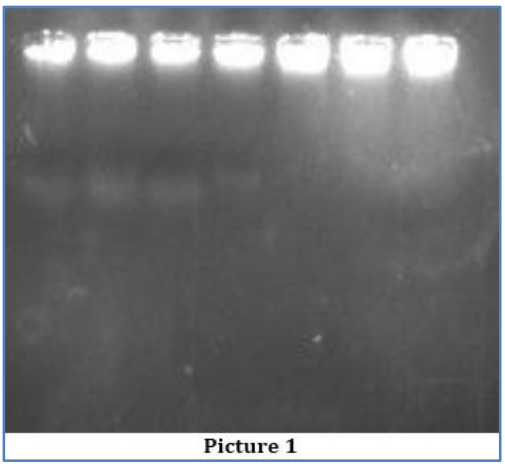
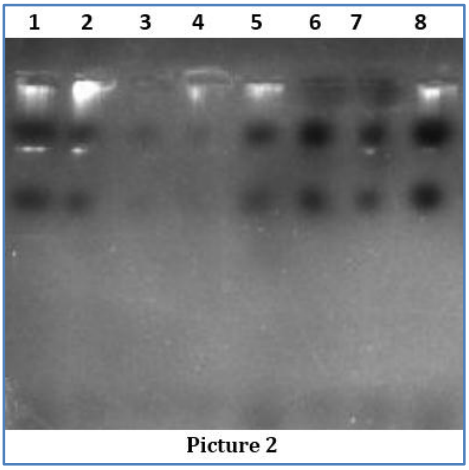


Picture 1



Picture 2

Picture 1: Representative gel picture of genomic DNA isolated from various tissue samples.

Picture 2: Representative gel picture of EBV gene target showing that EBV was not detected in any of the samples.

- Lane 1 & 2 represents EBV positive DNA (Positive Control).
- Lane 3 represents water.

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- Lanes 4-8 represent amplification of DNA from six different samples, all of whom lack any gene product.

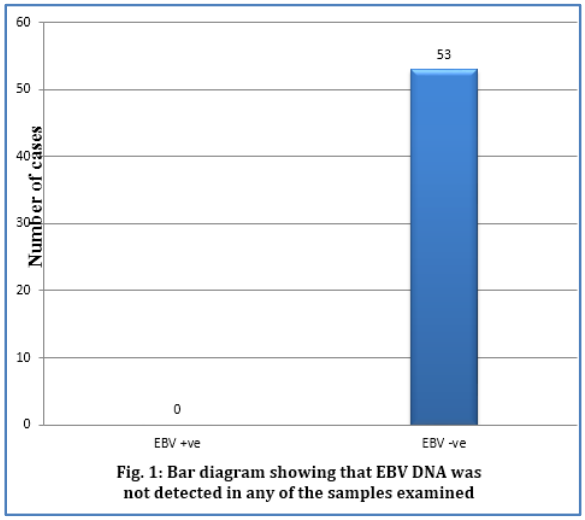

Fig. 1: Bar diagram showing that EBV DNA was not detected in any of the samples examined

Site of Disease	Number (Percentage)
Nasopharynx	4(7.54%)
Sinonasal	6(11.32%)
Oral Cavity	19(35.84%)
Tonsil	2(3.77%)
Pharynx	7(13.20%)
Larynx	15(28.31%)

Table 1: Distribution of Different Malignancies as Per Primary Site

Site	Subsite	Percentage
Sino nasal	Maxillary sinus	27%
	Nasal cavity	73%
Larynx	Supraglottic	60%
	Glottic	10%
	Infraglottic	30%
Oral cavity		
	Buccal Mucosa	20%
	Hard palate	8%
	Tongue	35%
	Gingiva	28%
	Retromolar trigone	9%

Table 2: Distribution of Malignancies as Subsite of Disease

DOI: 10.14260/jemds/2015/2047

Stage	Number (Percentage)
Stage 1	6(11.32%)
Stage 2	14(26.41%)
Stage 3	19(35.84%)
Stage 4	14(26.41%)

Table 3: Distribution of Patients as Per Stage of Disease

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FINANCIAL OR OTHER COMPETING INTERESTS: None

Date of Submission: 01/10/2015.
Date of Peer Review: 02/10/2015.
Date of Acceptance: 05/10/2015.
Date of Publishing: 12/10/2015.