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Detection of Epstein-Barr Virus in Oral Squamous Cell Carcinoma at Khartoum

Alfrazdag Mohamedahmed Abdalla Abdalrazig[1, *], Salah Eldin G Elzaki[2], Elneel Ahmed Mohamed[1]

[1]Department of Oral and Maxillofacial Surgery, Khartoum Teaching Dental Hospital, Khartoum, Sudan
[2]Institute of Tropical Medicine, Khartoum, Sudan

Abstract

Oral squamous cell carcinoma consider the most common type of oral cancer and carry poor prognosis especially in late stages, many factors like tobacco, alcohol, poor diet, have been investigated for their relation to this cancer however many patient develop oral cancer without exposure to these factors, this rise question about other factors may contribute to squamous cell carcinoma this factors include oncogenic viruses like human papilloma virus, herpes simplex virus and EPSTEIN-BARR VIRUS. Experimental cross-sectional study in Khartoum teaching dental hospital sudan and institute of tropical medicine, to detect presence of EPSTEIN-BARR VIRUS in oral squamous cell carcinoma samples using PCR, a total of 107 samples taken from oral squamous cell carcinoma patients 66 (61.7%) are male while 41 (38.3) are female. Mean age of the patient is 57. Forty four patients are snuffer while 63 are not. 17 patients are smoking while 90 patients are not. Only 7 patients consume alcohol. most of the patients are laborers and house wife. The patients came from all site of the Sudan with slight majority from central of the Sudan. DNA extracted and PCR used for detection. Total of 107 O.S.C.C samples tested by PCR for detection of EPSTEIN-BAAR VIRUS 35 (32.7%) samples are positive while 72 (67.3%) samples are negative. About 45 lesions in the lower jaw, 9 lesions in the lip, 20 lesions in buccal mucosa, 16 lesions in the upper jaw, 15 lesions in the tongue and only two lesions in the flour of the mouth. The 44 patients with snuffing habit show 20 patient positive for EPSTEIN-BAAR VIRUS while 63 non snuffer patients show 15 patients positive for EBV and this result show statistically significant different with P value (0.019). This study result revealed that EPSTEIN-BAAR VIRUS could be one of the causative factors that lead to squamous cell carcinoma especially in the patient with snuffing habbite.

Keywords

Oral Squamous Cell Carcinoma, EBV, PCR, Khartoum, Sudan

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1. Introduction

Oral cancer is usually defined as a neoplastic disorder in the oral cavity which includes the following areas: lip, buccal mucosa, lower and upper alveolar ridges, retromolar gingiva, oropharynx, floor of the mouth, hard palate, and the anterior two thirds of the tongue [1]

Most oral cancer is squamous cell carcinoma that originates from outgrowth of the mucosal epithelium [2].

According to an International Agency for Research on Cancer report: -(GLOBOCAN 2008), oral cancer is the tenth most common cancer for men and fourteenth for both sexes in the world. In terms of number of cases, accounting for approximately 480,000 new cases and mortality of 275,000 per year. In Sudan the incidence of oral cancer is 1547 and the mortality is 929 per year [3].

* Corresponding author
E-mail address: alfrzdagmohamedahmed@yahoo.com (A. M. A. Abdalrazig)

Many factors, including the habit of betel quid chewing and viral infections, tobacco, have been investigated for their association with the tumorigenesis of oral cancer [4].

However, some patients develop OSCC without exposure to these risk factors. This element suggests that additional causes, such as genetic predisposition, diet, or oncogenic viruses, may also help cells to override or escape the physiological mechanisms of proliferation control. [5]

The Epstein-Barr virus (EBV), unlike other members of the human herpes virus family, was the first human tumor virus identified [6] EPSTEIN-BARRVIRUS was initially seen by electron microscopy in lymphatic tissue culture cells derived from Burkett's lymphoma [7]. EPSTEIN-BARR VIRUS has been found in normal oral epithelium and in oral squamous cell carcinoma [8].

2. Material and Method

2.1. Study Site

Cross sectional hospital based study

This study was chosen to explore the presence of EPSTIN-BARR virus in patient with oral squamous cell carcinoma attending Khartoum dental teaching hospital in the period from August 2015 to August 2016.

2.2. Sample Collection

Small tissue biopsy nearly about 1 by 1 CM was taken from the lesion under local anesthesia and preserved in 1.5 ML appendegrof tube and absolute ethanol added and put in refrigerator at -1°C to 3°C.

Any sample is given a code number after the data collection sheet has been filled.

2.3. DNA Extraction

1 TISSUE SAMPLES PREPARATION: Any specimen sample divided into small pieces using sterile scalpel single scalpel for every sample to avoid contamination between the samples. Then the process started by adding phosphate buffer saline to wash out the ethanol. Then we restrictedly follow the steps of DNA extraction according to the VIVANTIS TECHNOLOGY instructions. As follow:-

2 TISSUE LYSIS: 250 µl of BUFFER TL and 20 µl of PROTEINASE K were added to the sample mixed thoroughly by pulse vortex till homogenous solution obtained then 12 µl of LYSIS ENHANCER added and mixed immediately then the sample incubated at 65°C for 3 hours.

3 HOMOGENIZATION: 2 volume (560 µl) of BUFFER CB (conditioning buffer) added to the sample and mixed thoroughly by vortex and incubate for 10 minutes at 65°C.

4 ADDITION OF ETHANOL: 200 µl of absolute ethanol added to the sample and mixed thoroughly by vortex.

5 LOADING TO COLUMN: approximately 600 µl of sample transferred into a column assembled in a clean collection tube (provided by manufacture) centrifuged at 8000 R\M for 1min. discarded flow through. (sediment was poured away) The same step repeated for the remaining of the sample.

6 COLUMN WASHING 1 the column washed with 500 µl of wash buffer 1 centrifuged at 8000 R\M for 1 min. discarded flow through.(sediment was poured away)

COLUMN WASHING 2 the column washed with 500 µl of wash buffer 2 centrifuged at 8000 R\M for 1 min. discarded flow through.(sediment was poured away) The column washed once again.

7 COLUMN DRYING: the column centrifuged at 12000 R\M for 2 minutes.

8 DNA ELUTION: the column was place in microcentrifuge tube 200 µl of preheated ELUTION BUFFER was added centrifuge for 1 min. at 5000 R\M to elute DNA.

2.4. Polymerase Chain Reaction

The polymerase chain reaction stared after addition EPSTEIN-BARR VIRUS primer to the DNA sample. In PELTIER. THERMAL CYCLER. After selection of specific program for the EPSTEIN-BARR VIRUS. The reaction started as follow:

GTGGTTTGTCCAAACTCATC reverse.

CCGCTCCTACCTGCAATATCA forward.

First denaturation at 95°C for 5 minutes.

Denaturation 95°C for 1 minute.

Annealing temperature 55°C for 1 and half minutes.

Extension at 72°C FOR 1 minute.

Final extension 72°C for 5 minutes.

Then all the samples tested in electrophoresis device to show which samples contain EPSTEIN-BARR VIRUS.

2.5. Ethical Clearance

An ethical approval was taken from research Ethics committee of Sudan medical specialization board.

Before biopsies were taken we explain to patients the nature and type and the aim of study and verbal consent have been taken.

About confidentiality any patient in this study give a number code and the patient name not appear in this study.

3. Results

Total of 107 biopsies were taken from patient with OSCC attending KDTH. Sixty six were males and 41 were females. Seventeen were smokers and 90 were not smokers. Snuff dippers were 44 and 63 were not snuff dippers. Only 7 were alcoholic consumers. As showed in the graph:

THE HABITS

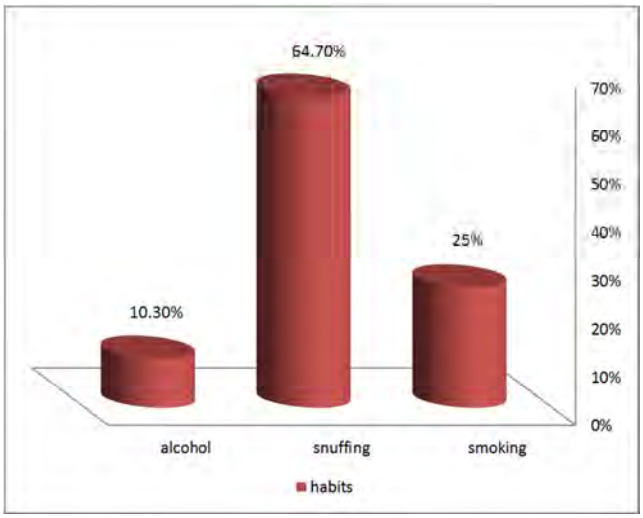

Figure 1. Seventeen patients were smokers, 44 patients were snuff dipper and Only 7 patients were alcoholic.

Forty five patients were having the O.S.C.C in their lower jaw while 20 patients in the buccal mucosa. upper jaw lesions was in 16 patients. 15 patients were having the lesions in their tongue. Lip O.S.C.C was in nine patients. only two lesion were occurred in the floor of the mouth. As figure show.

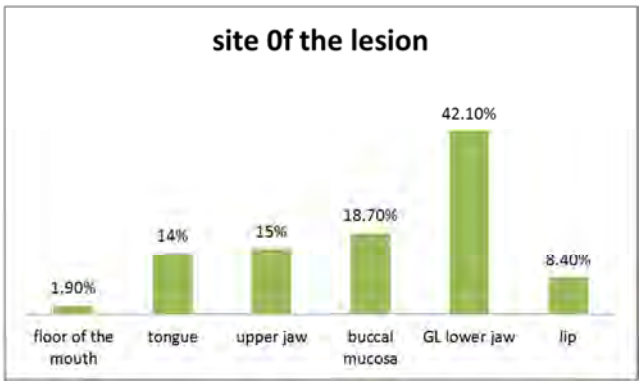

Figure 2. Shows most of the cancerous lesion located in gingivolabial area of the lower jaw (45), followed by the buccal mucosa (20), upper jaw (16), tongue (15) lip (9) and only 2 lesions occur in the floor of the mouth.

one hundred and seven samples were tested by PCR for the presence of EBV 35 samples were positive for EBV which represent (32.7%) while 72 samples were negative with percentage of (67.3%). As illustrated in the figure.

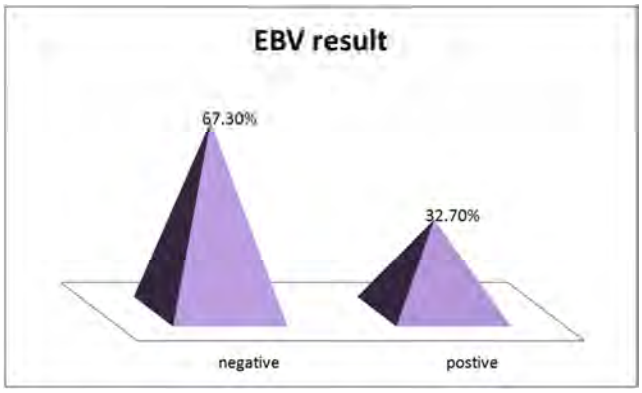

Figure 3. 35 samples (32.7%) are positive for EBV where 72 (67.3) are negative for EBV as shown in above figure.

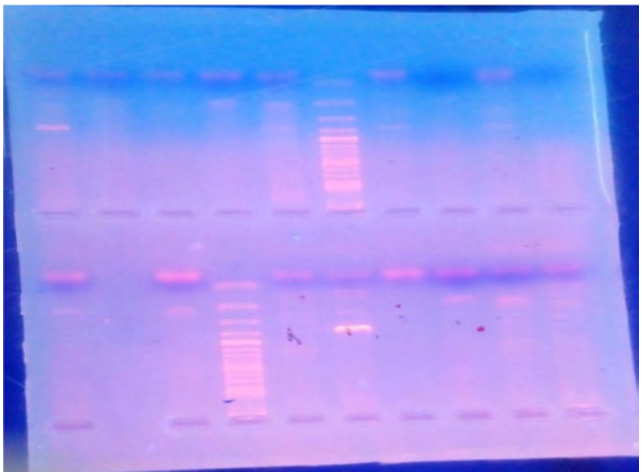

Figure 4. Examples of positive samples for EBV.

About two thirds of the positive samples were occurred in the patients with the snuff dippers habit this showed statistically significant difference with P value of (0.019) as appeared in the next tables:

Table 1. Displays statistically significant difference with p.v = 0.019. positive virus result appeared greater in snuffer patients than in non-snuffer.

Habit	NO	+ ve	-ve
Snuffer	44	20	24
No snuffer	63	15	48

4. Discussion

Cancer of the oral cavity is the sixth most common malignancy worldwide and account for approximately 5% of malignant tumors in developed countries. [9]

Many factors like habit of betel quid chewing and viral infection, tobacco, have been investigated for their association with the tumorigenesis of oral cancer. [10]

EBV has ability to distribute worldwide so it infects individuals in all societies and different places, [11], even individuals in the most remote and isolated tribes such as those in the Melanesian islands and Amazonian plateau. [12]

This study was done to explore presence of EBV DNA in biopsies taken from patient attending KTDH in the period from August 2015 to August 2016 (total coverage) PCR was used as the most of the same studies worldwide use the same methods and In situ hybridization methods.

This study showed that: its result as the international range of the others studies done in the last many decades to explore presence of EBV in the O.S.C.C, Which show virus prevalence ranging from (15% to 77%), [13].

In a study by Higa et al. 54 patients with oral squamous cell carcinoma in Okinawa compared with 21 and 20 patients from Kitakyushu and Kumamoto in Kyushu, mainland Japan, respectively. 39 patients (72.2%) were infected with EPSTEIN-BAAR VIRUS.

The authors concluded that in Okinawa, EBV infection was frequently demonstrated in oral squamous cell carcinoma (P0.001) however, in mainland Japan, there was no significant correlation between EBV and oral Squamous cell carcinoma. [14]

This study by Higa et al showed high positive result (72%) unlike the present study which show result of (32.7) this difference between the two studies most likely due to the technique of PCR procedure of Japan study (by Higa et al) which targeted many genetics materials of EPSTEIN-BAAR VIRUS not only the whole virus genome like present study.

The using of technique that targeting many genetics materials of EPSTEIN-BAAR VIRUS not only the whole virus genome justifies the high result of study carried out by Ching-Yu Yen et al. to detect EPSTEIN-BAAR VIRUS infection and gene expression in oral cancer in patients in Taiwan by microarray analysis. The majority of cases (82.5%) were EPSTEIN-BAAR VIRUS positive, This study targeted beside the EPSTEIN BAAR VIRUS DNA many virus genes like (EBNAs, LMP2A and 2B) [15]. while in the present study the positive result was (32.7) and this difference between the two study due to using of conventional PCR which targeted the whole virus genome in the present study. In contrast to the Ching-Yu Yen et al study which targeted many genetics materials of EPSTEIN-BAAR VIRUS so it was showed high positive result.

In study conducted by Sand et al. examining 29 patients with OSCC, 23 with OLP, and 67 with clinically healthy oral mucosa conventional nested PCR methods was used to detect EPSTEIN BAAR VIRUS DNA. Sand et al study showed this result; the prevalence of EPSTEIN BAAR VIRUS in patients with OSCC were (37.9%) and (26.1%) for those with OLP both percentages were statistically significant compared with that of control (703%), these result is closer to the present study since the both studies used the same method of PCR. Nested PCR used in study conducted by Sand et al. is slightly more sensitive than the outer PCR used in present study and this it may explain the slight different between the result of the two study (5%) [16].

The present study showed statistically significant result among patients with OSCC and snuffing habit (p 0.019) when compared with patients of OSCC and non-snuffer.

In study done by Shimakage et al using in situ hybridization probe targeting three different EPSTEIN BAAR VIRUS genome fragments. The BamHIW fragment appears positive in all cases. The EBER1 fragment appear positive in 16 (66%) out of 24 cases. The EBNA2 fragment appear positive in 9 (75%) out of 12 cases [17]. Shimakage et al Study showed high positive result not like present study which showed (32.7%) positive result. this difference between the two studies related to the structures targeted in each study. In the present study conventional PCR was used which targeted the whole virus genome while Shimakage study used in situ hybridization which targeted small pieces of the virus genome and this method is more sensitive than method used in present study.

There are other factors that may justify the difference between this study and the others studies that showed high positive result. These factors like sample size, preparation of the samples (contamination between the samples), methods of sample storage.

5. Conclusion

Inspite of improvement in medical care and diagnostic tools OSCC still consider a fatal disease with poor prognosis especially in advanced stages of disease. In this situation risk factors of this disease should be investigate carefully and any suspected factors also should be studied and tested for their possible roles in tumarogenesis of OSCC. However very important to study the viruses especially those associated with other head and neck malignancies. In this study 107 samples of O.S.C.C from 107 patients in K.T.D.H mean age of them were 57 years, 66 patients were male, 41 were female, 44 were snuffer 63 were not, 17 patients were smokers while 90 were not, only 7 patients were consume alchol. the presence of EBV in OSCC samples were tested by using conventional PCR showed 32.7% of cases appear positive for this virus while 67.3% of the samples were negative. this result reflect that EBV may play some role in development of OSCC especially in patient with another habits (co factor) like snuffing because the result show statistically significant difference in patient with snuffing habit (P value = 0.019).

Table 2. Chi-square tests.

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.514[a]	1	.019		
Continuity Correction[b]	4.575	1	.032		
Likelihood Ratio	5.480	1	.019		
Fisher's Exact Test				.023	.016
Linear-by-Linear Association	5.463	1	.019		
N of Valid Cases[b]	107				

The results of this study encourage further studies to determine the possible role of this virus in developing O.S.C.C like Experimental studies at the level of molecular biology to investigate the certain and degree of oncogenecity of EBV by incorporate the genome of this virus with the genome of experimental animals to see how this virus can induce tumors formation. Experimental studies to show that any relations between this virus and early detection or diagnosis of O.S.C.C and prognosis of the disease.

The role of some viruses in causes of O.S.C.C strongly confirmed like HPV so development of vaccine for this virus and all others suspected viruses may play role in reducing incidence of this disease.

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