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Detection of HSV-1 and EBER in Patients with Mucoepidermoid Carcinoma

KEYWORDS: Mucoepidermoid carcinoma, HSV-1, EBER, Immunofluorescence technique, in situ hybridization

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ABSTRACT *Background: Mucoepidermoid carcinoma is the most common type of salivary gland malignancy in children. It might be found in other organs, as bronchi, lacrimal sac and thyroid. However, different studies of viruses in mucoepidermoid carcinoma present conflicting results and some of these works remain in dispute. Objective: To assess the expression of herpes simplex virus-1 and Epstein Barr virus-encoded small RNA (EBER)using immunofluorescence technique and in situ hybridization in stage of mucoepidermoide. Materials and methods: Formalin-fixed paraffin embedded tissues from 22 patients with mucoepidermoide collected from dentist college hospital in Baghdad, In addition, ten apparently normal mucoid tissue autopsiesused as control group. Tissue blocks were sectioned and stuck on charged slides and used for the detection of HSV-1 and EBER. Results: The expression of HSV-1in patients' tissues with mucoepidermoid in the present study was 27.3%(6 out of 22).While EBER was 63.6% (14 out of 22), where significant differences was found between expression of HSV-1, EBER and age of patients with mucoepidermoid carcinoma, but there was no significant correlation associated between viral expression with gender and site of tumor. Conclusion: Herpes simplex virus-1 and Epstein Barr virus-encoded small RNA could be a co factor in the oncogenesis of mucoepidermoidor could infect cancer tissues opportunistically.*

Introduction

Mucoepidermoid carcinoma (MEC) is most commonly encountered in salivary glands but can also be seen at other locations, including bronchus, trachea, esophagus, breast, pancreas, and thyroid gland [1]. Salivary gland neoplasms are noted for their histological variability, reflecting the anatomy of these glands, benign and malignant salivary gland neoplasms may arise either from epithelial, mesenchymal, or lymphoid origins [2].

Herpesviruses comprise the largest family of viruses with oral manifestations. Eight types of herpesvirus are known to be pathogenic in human, with varying significance relative to oral diseases [3]. All herpisviruses are structurally similar. Each has an icosahedra core surrounded by a lipoprotein envelope. The genome is linear double-stranded DNA, the virion doesn't contain a polymerase [4].Herpes simplex virus-1(HSV-1) primarily causes mouth, throat, face, eye, and central nervous system infections, while HSV2 primarily causes anogenital infections. However, each may cause infections in all areas [5]. In all cases HSV is never removed from the body by the immune system. Following a primary infection, the virus enters the nerves at the site of primary infection, migrates to the cell body of the neuron, and becomes latent in the ganglion [6].

Epstein Barr virus is one of the viruses that have some unclear and controversial points in its ability to trigger the development of certain tumors [7]. Like Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, gastric carcinoma and post-transplant lymphoproliferative disease [8]. The small untranslated RNAs EBER-1 and -2 are accumulated at high levels during all forms of latency and regulate apoptosis through different mechanisms. EBER-1 interacts with the interferon-inducible protein kinase R (PKR), and inhibits its activation by double-stranded RNAs, protecting infected cells from IFN-induced apoptosis [9].To assess the expression of herpes simplex virus-1 and Epstein Barr virus-encoded small RNA (EBER) using immunofluorescence technique and *in situ* hybridization in stage of mucoepidermoide.

Materials and Methods

Samples Collection: Twenty two cases of MEC were retrieved from the archives of oral pathology laboratories of Medical College of Dentist-University of Baghdad, 14 patients were men and8 were women, ranging in age from 36 to 65 years. In addition, ten apparently normal mucoid tissue autopsiesused as control group were included in this study, specimens were divided according to site of infection into four groups: Parotid (n=4), Palate (n=8), Submandibular(n=6) and Buccal mucosa (n=4the patients samples were collected during the period from October-2012 till February-2013.

Tissue processing: Hematoxylin and eosin-stained slides were reviewed in all cases; unstained paraffin sections for detection herpes simplex virus-1 Glycoprotein C which target of HSV-1 Ag (United States biological, Cat. No. H2033-08E). By used direct immunofluorescence analysis and Epstein Barr Virus-encoded small RNA (EBER) (ZytoVision GmbH. Fischkai 1D-27572 Bremerhaven. Germany) and detection kit of EBER by*in situ* hybridization analysis.

Direct Immunofluorescence: US Biological herpes simplex virus-1 Glycoprotein C kit was used for detection of HSV-1 Ag by direct immunofluorescence assay according to manufacturer's protocol. The slides were deparaffinizedby xylene and rehydrated dehydrated by graded alcohol concentration (100%, 95% and 70%) and distal water. The slides were rinsed 3 times with cool phosphate buffer saline (PBS), and left to dry at room temperature, then blocked with blocking buffer (1-2% Bovine Serum Albumin) at room temperature for 2 hours. The slides were washed and left to dry at room temperature. All slides were treated with fluorescent-tagged primary antibody (dilution 1:10 with blocking buffer), then incubated overnight in refrigerator at 4˚C. The slides were washed, and then examined by used fluorescence microscopy. The slides were considered as a positive when the specimen contained one or more cells displaying HSV-1 specific fluorescence (apple-green fluorescence) and considered negative when there is no specific fluorescence. In each run used two types of controls, Positive and negative controls. Positive control, Consisted of two patients having infection with herpes labialis, and a swab were taken from the site of infection and was put in a charged slide and the same procedure for immunofluorescenc (IF) was done. Negative control: Two slides were prepared as the procedure of IF to the whole samples but one slide was prepared by putting sample without using the substrate, but instead of that we used the

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bovine serum albumin, while the other slide was prepared by using distilled water instead of the sample.

***In situ* hybridization:** The 4µm thick paraffin sections were deparaffinized by xylene and dehydrated by graded alcohol concentration (100%, 95% and 70%) and distal water then treated with pepsin solution for 20-30 min at 37°C in a humidity chamber. Immerse slides in distilled water and blot off the water and then with digoxigenin-labeled probe with EBER. Denature the slides at 95°C for10 minutes on a hot plate. Transfer the slides to a humidity chamber and hybridization was the carried out for 2 hours at 37°C for RNA-targeting probes. It is essential that the tissue/cell samples do not dry out during the hybridization. The slides were soaked in Wash Buffer TBS for 5 min to remove the cover slip, and then treated with AP-Streptavidin. One to two drops of NBT/BCIP were placed on tissue section and incubate for 30 minutes at 37°C in a humidity chamber; the latter was monitored by viewing the slides under the microscope. Colored precipitate will form at the site of the probe in positive cells. Slides were then counterstained using eosin and sections were mounted with a DPX. Finally Evaluation of the sample material is carried out by light microscopy by a pathologist at power 400.

Data analysis: Fisher's exact test and t-test were used to obtain statistically significant differences between two groups with ($p<0.05$) being considered statistically significant.

Results

Minimum age was 37 years and maximum was 65 years (mean range = 45.45 years) as showed in (Table 1).According to age stratum, there was highly significant differences noticed between three age groups, 36-46 years was the higher percent (72.7%), while 58-68 years was the lower percent (9.1%) (Table 2), majority of cases were male (63.6%) while female showed (36.4%) (Table3). More ever, specimens were divided according to site of infection into four groups: Parotid (n=4), Palate (n=8), Submandibular (n=6) and Buccal mucosa (n=4) (Table 4).

Table (1): Distribution of mucoepidermoid carcinoma patients according to the age

Studied groups	Number	Age/ years			(t-test)/ P-value*
		Mean	Mini	Maxi	
Control	10	46.5	37	58	P>0.05
MEC	22	45.4	36	65	

*Non-significant

Table (2): Distribution of Mucoepidermoid carcinoma patients according to their age strata

Age stratum	Number	Percentage	Comparison of Significance Chi²-value Sig.
36-46 47-57	16 4	72.7% 18.2%	23.42 P<0.0001
58-68 Total	2 22	9.1% 100%	

*Significant

Table (3): Distribution of mucoepidermoid carcinoma patients according to the gender

Gender	Number	Percentage	Comparison of Significance Chi²-value Sig.
Male Female	14 8	63.6% 36.4%	3.26 0.071
Total	22	100%	

*Non-significant

Table (4): Distribution of mucoepidermoid carcinoma patients according to the Site of infection

Organ	Number	Percentage	Comparison of Significance Chi²-value Sig.*
Parotid Palate	4 8	18.2% 36.4%	2.67 0.445
Submandibular Buccal mucosa Total	6 4 22	27.3% 18.2% 100%	

*Non-significant

The interested results demonstrated that 6 cases (27.3%) of mucoepidermoid carcinoma were positive for HSV-1, while 14 cases (63.65%) were positive for EBER. On the other hand, the statistical analysis was demonstrated a highly significant difference in HSV-1 and EBER expression among patients with mucoepidermoid carcinoma when compared with healthy control group (Table 5).In the present study, as shown in (Tables 6, 7) there was no significant differences between expression of HSV-1,EBER and different variable like age, gender and site of tumor, most cases occur in the age group 36-46 years, also males was more than females but statistical analysis did not show significant differences at P>0.05and according to the site of infection palate constituted the higher percent, the result of immunofluorescence revealed that signal within infected tissue as showed in figure 1-B, according *in situ* hybridization figure 2-A showed negative result while B showed positive signal within nucleus of infected cell.

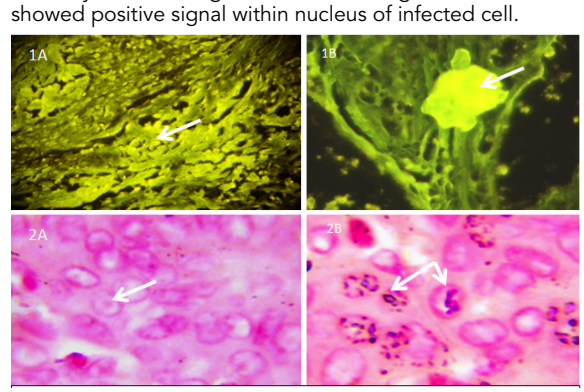

Figure (1): Direct Immunofluorescence in mucoepidermoid carcinoma, A- Negative expression B- positive expression. Figure (2): In situ hybridization in mucoepidermoid carcinoma, A- Negative expression B- positive expression.

Table (5): The percentage of the HSV1, EBER positive and negative tests results in the studied groups

Organ	Control	MEC	Comparison of Significance Chi²-value Sig.
Positive HSV-1 Negative HSV-1	0 10	6(27.3%) 16(72.7%)	0.067 Non-Sig.
Positive EBER Negative EBER	0 10	14(63.6%) 8(36.4%)	0.001 Sig.*

*Significant

Table (6): Immunoflouresent positive and negative HSV-1 and related with different parameters

Variables Negative		Expression of HSV-1		Comparison of Significance	
		positive	p-value	Sig.	
Gender	Male	16(80%)	4(20%)	0.055	0.815
	Female	10(83.3%)	2(16.7%)		

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Age	36-46	14(70%)	6(30%)	4.431	0.109
	47-57	9(100%)	0		
	58-68	3 (100%)	0		
Type of organ	Parotid	4(100%)	0	6.838	0.145
	Palate	6(75%)	2(25%)		
	Submandibular	4(66.6%)	2(33.3%)		
	Buccal Mucosa	2(50%)	2(50%)		

*Non-significant

Table (7): ISH positive and negative EBERand related with different parameters

Variables Negative		Expression of EBER		Comparison of Significance	
		positive	p-value	Sig.*	
Gender	Male	14(70%)	6(30%)	4.097	0.043
	Female	4(33.3%)	8(66.7%)		
Age	36-46	10(50%)	10(50%)	2.653	0.265
	47-57	7(77.8%)	2(22.2%)		
	58-68	1 (33.3%)	2(66.7%)		
Type of organ	Parotid	0	4(100%)	14.392	0.006
	Palate	4(50%)	4(50%)		
	Subman-dibular	2(33.3%)	4(66.6%)		
	Buccal mucosa	2(50%)	2(50%)		

*Non-significant

Discussion

The presence of HSV-1 was investigated in the present study. Six of 22 cases (27.3%) of mucoepidermoid carcinoma were positive for herpes simplex virus-1 Glycoprotein C by direct immunofluorescence. This result was in agreement with the finding of Melnicket *al*. [10], who determined the presence of cytomegalovirus (CMV) in adults, with peak incidence from 20-40 years of age. Mucoepidermoid Carcinoma is most commonly encountered as a tumor of salivary glands; it has been described in the major salivary glands and in the minor glands, particularly in the oral cavity [11].

On the molecular level, HSV-1 consists of more than 80 genes that are expressed sequentially in a strongly regulated cascade [12, 13]. Apoptosis of host cells represents an important defense mechanism against viral invasion by preventing viral replication and dissemination. The extrinsic pathway of apoptosis induction is triggered by ligation of death receptors [14] or by injection of granzymes [15]. Intrinsic triggers of apoptosis such as DNA damage, oxidative stress, deprivation of growth factors, and viral infection disrupt the integrity of the mitochondrial membrane, resulting in release of cytochrome c into the cytoplasm [16]. The results of Nguyen*et al*.[17] indicated that apoptosis has been associated with herpes simplex virus 1 (HSV-1) latency and diseaseseverity. There is an intricate balance between pro- and anti-apoptotic processes during HSV-1infection. When anti-apoptotic pathways are suppressed, this balance is upset and the cells die by apoptosis, who referred to as HSV-1-dependent apoptosis (HDAP). It has been observed previously that HeLa cancer cells exhibit an enhanced sensitivity to HDAP.

Regarding the HSV-1 expression the results positive rate revealed that the prevalence of HSV-1 was higher in age group 36-46 (30%) than others as recorded. This may be related to the rising incidence with age may be explained by the accumulation of somatic mutations associated with the emergence of malignant neoplasms. In addition, the observed impairment in the immune system in such ages, due to senescent decline in the immune surveillance, might lead to accumulation of cellular DNA mutation that could be regarded as an additional significant factor in the development of such malignancies [18]. Also, the thymus function is known to decline with age. The thymus reaches its maximal size at puberty and then atrophies, with a significant decrease in both cortical and medullary cells and an increase in the total fat content of the organ. Whereas the average weight of the thymus is 30 grams in human infants, its age-dependent involution leaves an organ with an average weight of only 3 grams in the elderly [19]. Moreover, may be related with decline in the number of NK, which play an important role in early natural surveillance against cancer and infectious disease, a progressive age-related shift in the circulating lymphocyte population from conventional T cells to NK cells [20].

Regarding the gender, the results revealed that the positive rate of HSV-1 was higher in male than female, but statistical analysis didn't show revealed significant correlation. These results were in agreement with study of Kumar et al.[21],who indicated that males and females are affected about equally, which usually in the 6th or 7th decade of life. In the parotids of 70% to 80% of these tumors are benign, whereas in the submaxillary glands only half are benign thus it is evident that a neoplasm in the submaxillary glands is more ominous than one in the parotids

According to site of cancer, the present study demonstrated that most positive HSV-1 cases occurred within palate and submandibular. These results are agreed with the findings of Soames and Southam [22], who indicatedthattumors of the salivary glands constitute an important area in the field of oral and maxillofacial pathology. They are relatively uncommon with an annual incidence in the western world of about 3 per 100 000 population. Tumors of the major salivary glands are far more common than those of the minor salivary glands which account for only about 15-20 percent of all salivary gland tumors. About 90 percent of the tumors in major glands usually occur in the parotid gland and 10 percent in the submandibular gland, while sublingual gland tumors being rare. About 55 percent of minor salivary gland tumors arise in the palate and about 20 percent of cases in the upper lip, with the remainder scattered throughout the mouth.

This study subjected twenty two formalin-fixed, paraffin-embedded archival tissue blocks with MED carcinoma to a recent generation of ISH technique to detect the EBER, The results of this study appear to 14 out of 22 cases (63.6%) of MED carcinoma were positive for EBER. These findings agree with [23], who indicatedthe etiology of salivary gland tumors is unknown, the involvement of environmental and a genetic factor has been suggested. Radiation exposure has been linked to the development of the benign warthin tumor and to the malignant mucoepidermoid carcinoma. Epstein-Barr virus may be a factor in the development of lymphoepithelial tumors. Genetic alterations, such as allelic loss, monosomy, polysomy and structural rearrangement, have all been studied as factors in the development of salivary gland tumors.

The association of mucoepidermoidcarcinoma might development with the age. Notable, no significant association has been shown between EBER expression and age of patients that could be explained by the prolonged chance of exposure to environmental carcinogens such as chemicals, radiation and viruses, which were regarded as important promoting factors in the development of oral cancers [24].

Regarding the study of the gender distribution in positive EBER result, it was found that 6 cases (30%) of MED were males while the rest 8 cases (66.7%) were females. These findings are statistically not significant. This may be related with male and female have the same chance for exposure to carcinogenesis. On studying the site distribution of MED, the present study had found that the parotid, palate and submandibular were the most affected site (4 out of14), (4 out of 14), (2 out of 14) respectively, and lastly the buccal mucosasite is lowest (50%).This result might be attributed to the differences in the size of study samples as well as to the methods used for collection them. In conclusion, herpes simplex virus-1 andEpstein Barr virus-encoded small RNA could

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be a cofactor in the oncogenesis of mucoepidermoidor could infect cancer tissues opportunistically.

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