The Frequency of Epstein-Barr Virus Infection As A Pathogenic Agent In Laryngeal Carcinoma of Iraqi Patients Demonstrated By LMP-1 Expression

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Abstract

The frequency of Epstein-Barr Virus (EBV) antigen; latent membrane protein-1 (LMP-1) expression in cases of laryngeal carcinomas was investigated by immunohistochemical staining technique. Fifty-six cases were included in this work; all cases of laryngeal carcinomas were squamous cell carcinomas with different degrees of differentiation. Immunohistochemical staining results revealed 60.7% positive staining results in overall cases, distributed according to the degree of differentiation almost equally (60% in well differentiated, 64.5% in moderately differentiated and 60% in poorly differentiated). The concordance of cigarette smoking with LMP-1 expression was high (96%). These results indicate the highly possible oncogenic effect of EBV infection on the development of laryngeal carcinomas in Iraqi patients.

Key words: ***Laryngeal carcinoma, EBV, LMP-1***

الخلاصة

في هذا البحث تم الاستقصاء عن عدد مرضى سرطان الحنجرة المصابين بفايروس EBV من خلال التعرف على احد المستضدات الخاصة به (Latent Membrane Protein-1: LMP-1) باستخدام تقنية المعلمات المناعية النسيجية. تم ادراج ست و خمسين حالة من حالات سرطان الحنجرة في هذا البحث. كانت كل الحالات من نوع السرطان الخلايا الحرشفية مع درجات مختلفة من التمايز. أظهرت نتائج استخدام تقنية المعلمات النسيجية المناعية نسبة ايجابية في 60,7% في مجمل الحالات، مع نسبة متشابهة تقريباً لجميع درجات التمايز (60% للتمايز الجيد، 64,5 % للحالات متوسطة التمايز و 60% للحالات ضعيفة التمايز). كما أظهرت الدراسة نسبة عالية من التزامن بين التدخين و الاصابة بفايروس EBV من خلال التعرف على مستضد LMP-1 البالغة نسبتها 96%. هذه النتائج تشير إلى التأثير المسرطن المحتمل لفايروس EBV في نشوء سرطان الحنجرة في العراق.

الكلمات المفتاحية : سرطان الحنجرة، فايروس EBV، مستضد LMP-1.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpes virus which causes infectious mononucleosis and is associated with human cancers such as Burkitt's lymphoma (Jiwa *et al.*, 1995), nasopharyngeal carcinoma (NPC) (Vera-Sempere *et al.*,1996), and lymphomas (Jiwa *et al*, 1995; Pallesen *et al*, 1991; Murray *et al.*, 1992). EBV is normally found as a widespread asymptomatic infection with >95% of the world's adult population being lifelong carriers. The virus genome is a doublestranded DNA molecule of approximately 172kb encoding at least 80 proteins. Two strains of EBV exist, although the bulk of the genome appears to be identical (Baumforth *et al.*, 1999; Cruchley *et al.*, 1997). EBV-infected cells and neoplasms generally show one of three different expressional patterns of latency-associated viral genes. In latency type I, only EBV-encoded-nuclear antigen 1 (EBNA-1) is expressed together with two small polyadenylated nuclear RNAs (EBER 1 and 2) observed in Burkitt's lymphoma. In latency type II, additional expression of three latent membrane proteins, LMP-1, LMP-2A and LMP-2B are observed, seen in Hodgkin's disease and nasopharyngeal carcinoma. Latency type III is seen in lymphoprotiferative diseases arising in immunocompromised individuals and EBV-transformed lymphoblastoid cell lines. In this group all six EBNAs (EBNA-l/2/3A/3B/3C/LP), all three LMPs and the two EBERs are expressed (Cruchley *et al*).

In parts of the world with high prevalence of NPC, serological studies indicating elevated immunoglobulin A and G antibodies to virus-encoded antigens, virus capsid antigen, and early antigen detected in sera and throat washing samples from patients with NPC are regarded as tools to confirm the diagnosis (AS Abdulamir *et al*, 2008; Kee-Ching *et al.,* 1994). EBV DNA has been detected in biopsy specimens from patients with NPC and other head and neck tumors. Furthermore, EBV-encoded nuclear antigen1 (EBNA-1) is found in 100% of tissues from patients with NPC; latent membrane protein (LMP) is found in 65% of the biopsy specimens from patients with NPC. Biologically, EBNA-1 is an EBV-encoded transactivator and is required in *trans* to maintain the virus genome in an episome form. On the other hand, the LMP gene (BNLF-1) has a profound effect on the morphology of rodent cells, indicating that the protein is potentially oncogenic. In addition, the BNLF-1 gene is expressed abundantly in African NPC cells (C15), suggesting its oncogenic role in NPC. Recent reports show that there are two specific EBV variants, the Taiwan and CAO strains isolated from patients with NPC in Taiwan and the People's Republic of China, respectively. The LMP gene (BNLF-1) of the Taiwan variant strain is shown to possess recurrent mutations in biopsy specimens from patients with NPC and is different from the BNLF-1 sequence of the B95-8, Jijoye, and C15 strains of EBV but is similar to that of the CAO strain (Kee-Ching *et al.,* 1994).

Laryngeal Carcinoma: Carcinoma of the larynx accounts for 2.2% of all cancers in men and 0.4% in women. Most patients are in their fifth decade of life or beyond, but cases occurring in much younger patients are on record (Lee *et al*, 1987; Mendez *et al.,* 1985). About 96% of the patients are males. Smoking is the main risk factor, this risk being enhanced by heavy alcohol consumption (DeStefani *et al*, 1987; Muscat *et al.,* 1992). Interestingly, a difference in the incidence of the various topographic sites within the larynx has been found depending on geographic location (Barnes *et al.,* 1986). Hoarseness is a common early symptom for glottic tumors but not for those located elsewhere. Anatomically, carcinomas of the larynx can be classified according to its location into glottic (60% to 65% of all cases), arising from the true vocal cord, supraglottic (30% to 35% of all cases); involve the false cord, the ventricle, and/or the laryngeal or lingual surface of the epiglottis, transglottic (less than 5% of all cases); those tumours cross the laryngeal ventricle and infraglottic (subglottic) (less than 5% of all cases). Under this category are included cancers involving the true cord with a subglottic extension of more than 1 cm as well as tumors entirely confined to the subglottic area. Microscopically, most carcinomas (around 90%) are of squamous cell type, with different degrees of differentiation. The rest represent less common variants including verrucous carcinoma, small cell carcinoma, basaloid squamous cell carcinoma and adenocarcinoma.

LMP-1: The latent membrane protein, LMP-1, is encoded by the *BNRF1* gene and contains 386 amino acids with a molecular mass of 63 kDa. It is regarded as an oncogenic protein as it is implicated in at least four transcription factor signalling pathways, and induces expression of multiple cell surface markers and cell adhesion molecules (Murray *et al*, 1992; Baumforth *et al.,* 1999). In Western blotting with lysates of EBV-transformed lymphoblastoid cell lines, anti-LMP-1 antibody labels a major band of 57-66 kDa depending on the virus isolate, sometimes accompanied by a minor band of 50-55 kDa corresponding to full length and truncated form of LMP. The antibody recognizes 20 geografically distinct EBV isolates (Rowe *et al.,* 1987). The antibody cross-reacts with a 43-44 kDa doublet of uncharacterized normal cell proteins in cellular extracts including that from EBV-negative control cell lines (Rowe et al).

Objectives

1. To illustrate the possible pathogenetic role of EBV infection in the development of laryngeal carcinoma in Iraqi patients.
2. To correlate other risk factors with EBV infection.
3. To demonstrate LMP-1 tissue expression in correlation with the degree of differentiation and anatomical location within the larynx.

Materials And Methods

Fifty six formalin fixed paraffin embedded tissue samples prepared from biopsies of laryngeal carcinoma were included in this study, they were randomly retrieved from private laboratories in Hilla, Iraq, during the period extending from 2008-2011. Histological sections were prepared to assess grade of differentiation. Other sections were stained using immunohistochemical staining procedure with monoclonal primary antibody to LMP-1, purchased from DAKO Inc. using envision staining technique. Statistical analysis were made using Kruskal-Wallis test

Results

The following table illustrates the histological grading of laryngeal carcinoma, based on the traditional three-tired scheme (well, moderately and poorly differentiated). This table also illustrates the rate of LMP-1 expression of neoplastic cells in each histological grade.

Table 1: The histological grades of laryngeal carcinomas in association with LMP-1 expression.

Grade	*Histological*		*LMP-1*	
	No.	*%*	*No.*	*%*
Well differentiated	35	62.5	21	60
Moderately differentiated	16	28.57	10	64.5
Poorly differentiated	5	8.92	3	60
Total	56	100	34	60.7

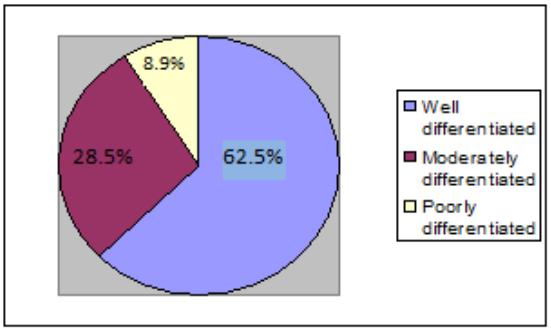


Fig. 1: A. The frequency of laryngeal squamous cell carcinoma according to histological grades. B. The frequency of LMP-1 expression in relation to the degree of differentiation.

The above table also illustrates the overall LMP-1 positive staining results in laryngeal carcinoma cases of this study to be 60.7%, representing 34 cases out of 56.

There was no significant statistical difference between grade of differentiation and LMP-1 expression ($p > 0.05$).

Table 2 shows the grade of LMP-1 expression distributed according to positive staining of neoplastic cells as: negative, + (<10% of cells), ++ (10-30% of cells) and +++ (> 30% of cells).

Table 2: The grade of LMP-1 expression in correlation with the grade of laryngeal carcinomas.

Grade	*Negative*		+		++		+++	
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Well differentiated	14	40	6	17.1	13	37.1	2	5.7
Moderately differentiated	6	37.5	4	25	5	31.2	1	6.2
Poorly differentiated	2	40	3	60	0	0	0	0
Total	22	39.3	13/34	38.2	18/34	52.9	3/34	8.8

Table 3: Illustrates the association of LMP-1 expression with the anatomical sites of laryngeal carcinomas.

Anatomical site	*Total*	*LMP-1 Expression*							
		Negative		+		++		+++	
		No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
Glottic	38	18	47.3	12	31.5	6	15.7	2	5.2
Supraglottic	5	2	4	1	2	2	4	0	0
Transglottic	3	0	0	0	0	3	100	0	0
Infraglottic	1	1	100	0	0	0	0	0	0
Undetermined	9	1	11.11	3	33.33	4	44.44	1	11.11
Total	56	22	39.3	13	47.05	18	44.11	3	8.8

Table 3 illustrates the anatomical distribution of different cases segregated into: glottic, supraglottic, transglottic, infraglottic and undetermined site (because of inadequate clinical data) in correlation with LMP-1 expression. As shown in the table, the majority of cases were glottic.

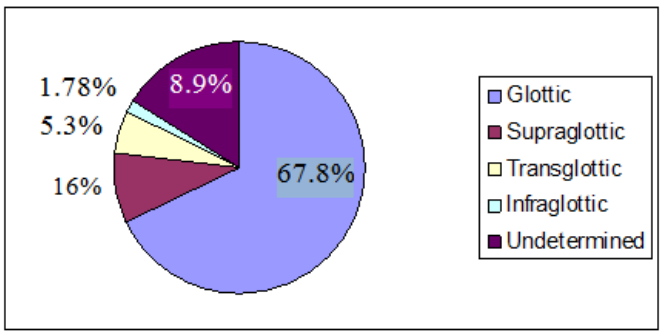


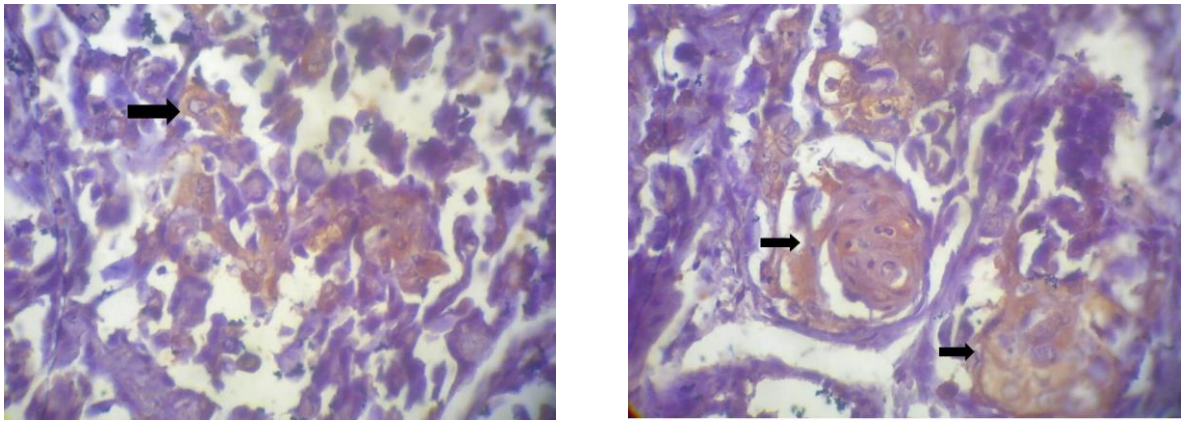
Figure 2: The frequency of laryngeal carcinomas according to the anatomical sites.

Table 4 shows the association of LMP-1 expression with cigarette smoking, being the well-known recognizable risk factor, in correlation with histological grade. In general, the total number of smoker patients with laryngeal carcinoma in this study was 52 out of 56 (92.8%). The number of cigarettes per day was not included in this study because of the deficiency in submitted clinical data.

There was no statistical correlation between smoking and LMP-1 expression (p: 0.816), but there was a good correlation with the total cases of laryngeal carcinoma (p: 0.033).

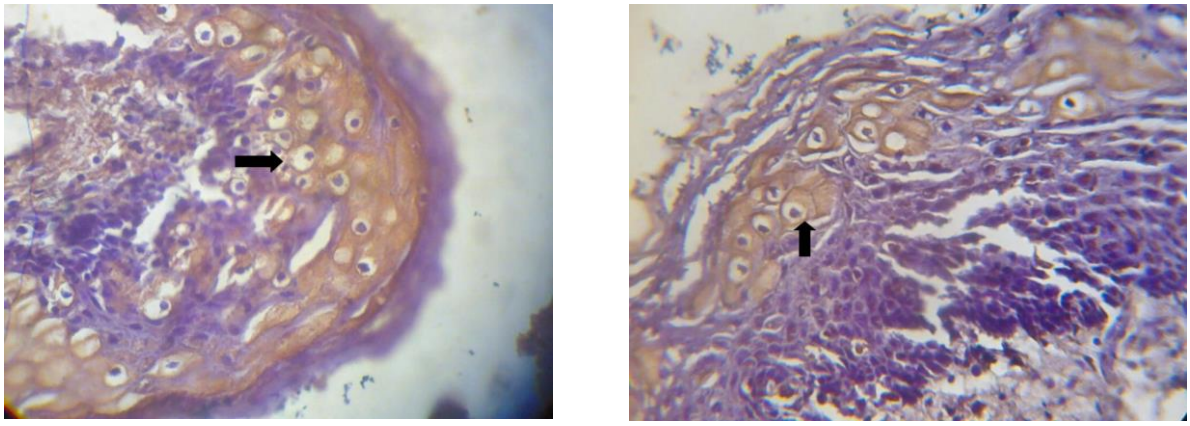
Table 4: Illustrates the association of cigarette smoking with LMP-1 expression, in different grades of laryngeal carcinomas.

LMP-1	*Negative*				+				++				+++			
Grade	*No.*	*%*	*Smoking*		*No.*	*%*	*Smoking*		*No.*	*%*	*Smoking*		*No.*	*%*	*Smoking*	
			No.	*%*			*No.*	*%*			*No.*	*%*			*No.*	*%*
Well differentiated	14	40	13	92.8	6	17.1	6	100	13	37.1	12	90	2	5.7	2	100
Moderately differentiated	6	37.5	5	83.33	4	25	3	75	5	31.2	5	100	1	6.2	1	100
Poorly differentiated	2	40	2	100	3	60	3	100	0	0	0	0	0	0	0	0
Total	22	39.3	20/22	90.9	13/34	38.2	12/13	92.3	18/34	52.9	17/18	94.4	3/34	8.8	3/3	100



A B

Fig. 3: Sections from two cases stained with anti-LMP-1, showing moderate expression in areas of squamous cell carcinomas. x400.



A B

Fig. 4 A&B: Show diffuse membranous and cytoplasmic positive staining with LMP-1 involving apical cells of an area adjacent to squamous cell carcinoma. These sections also show proliferative changes with cytologic atypia and cytoplasmic ballooning. x400.

Discussion

Results of this study show that there is an increased risk of development of laryngeal carcinoma after infection with EBV with an overall LMP-1 positive staining results of 60.7%. There was no significant difference in the LMP-1 expression in different grades of laryngeal carcinomas (60% for well differentiated, 64.5% for moderately differentiated and 60% for poorly differentiated). The results of this study correlates with reports from other parts of the world. Gök U *et al* reported the presence of EBV viral DNA in 11 squamous cell carcinomas of the larynx in Turkish patients out of 22 (50%), but only in 7 patients with vocal cord nodules out of 17 (41.2%), with no significant increase in the risk (Gök *et al.,* 2003).

Similarly; Kiaris H et al (Athens, Greece) investigated the presence of EBV viral DNA in laryngeal carcinoma and adjacent normal tissue, using PCR and RFLPs and revealed that 9 of 27 (33%) specimens harbored the EBV genome in the tumor tissue while only 4 (15%) specimens from adjacent normal tissue exhibited evidence of EBV infection. Three were EBV positive for both normal and tumor tissue. No association has been found with disease stage, histological differentiation and nodes at pathology (Kiaris *et al.,* 1995).

Liu Z et al investigated the expression of LMP-1 in 90 laryngeal specimens taken from laryngeal carcinoma of Chinese patients. LMP-1 was detected in 41 out of 90 (45.5%) laryngeal squamous cell carcinoma, among them the positive rates of poorly differentiated, moderately differentiated and well differentiated squamous carcinoma were 44% (12/27), 52% (25/47) and 26.6% (4/15) respectively. By this they regarded that there is a significant increase in the risk of development of laryngeal carcinoma with EBV infection (Liu *et al.,* 1997).

On the contrary of the above reported results, de Oliveira *et al* (State University of SãoPaulo, Botucatu, São Paulo, Brazil) demonstrated the presence of HPV viral DNA in 37.3% of his 110 cases, and none of them harbored EBV viral DNA (Deilson *et al.*2006). Similarly, Robson et al (Boston, Massachusetts) studied 4 children patients with laryngeal carcinomas demonstrating no EBV DNA by FISH technique (Robson *et al.,* 2010). Two cases of lymphoepithelioma-like carcinoma of the larynx were studied by Tardío JC et al (Spain) revealing no evidence of EBV infection immunohistochemically and by FISH technique (Tardío *et al.,* 1997).

In the above reports, compared with our results, it seems that there is a wide range of expression of EBV viral proteins and presence of viral DNA in laryngeal carcinomas of different populations all around the world, tending to be more prevalent in Eastern population (Chinese and Middle East) and disappear in Western populations. This may be attributable to different viral strains and different carcinogenic effects. In parts of the world endemic with EBV with more prevalence of nasopharyngeal carcinoma, like in China and South East Asia, throat swabs from normal population revealed EBV viral DNA with indicators of active viral disease (Kee-Ching G *et al.,* 1994). No such evidence has been found in Western population.

Conclusion

Laryngeal carcinoma and EBV infection seem to be pathogenetically associated in Iraqi patients. Although smoking is still the main risk factor, but infection with EBV seems to increase the risk of development of laryngeal carcinoma, despite the statistical insignificance.

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