Epstein–Barr virus in head and neck squamous cell carcinoma

A.H. Kamel,[1] M.A. El-Barawy,[1] M.H. Hashish[1] and S.M. El-Sheikh[2]

فيروس إبشتين–بار في السرطانة حرشفية الخلايا في الرأس والعنق

عزيزة حسن كامل، محمود عباس البراوي، منى حسن حشيش، سحر محمد الشيخ

الخلاصة: أجرينا مقارنة لخمسين مريضاً مصاباً بسرطانة حرشفية الخلايا في الرأس والعنق مع 45 من الشواهد من الأصحاء. وقد أخذت عينات من الخزعات المأخوذة من الأورام ومن النسج السوية للحالات والشواهد، وأجريت مقاطع من البرافين الذي غمرت فيه هذه العينات وعولجت لكشف المستضدات لفيروسات إبشتين–بار. وقد تم كشف بروتينات فيروسات إبشتين–بار لدى 38% من الحالات. و لم يكشف لدى أي من الشواهد الأصحاء، مما يشير إلى ترابط إيجابي. وقد تم إجراء اختبار لعينات من المصل لكشف الغلوبولينات المناعية الخاصة بفيروسات إبشتين–بار باستخدام طريقة مقايسة الممتز المناعي المرتبط بالإنزيم (إليزا) للمقارنة مع الموجودات بالفحص الكيميائي والهيستولوجي المناعي. ولوحظ أن المرضى الذين كانت لديهم نتائج التلوين الكيميائي الهيستولوجي المناعي إيجابية، كان لديهم أيضا عيارات مرتفعة وسطياً للأضداد، إذا ما قورنت بمن كانت لديهم نتائج التلوين الكيميائي الهيستولوجي المناعي سلبية. إن مقايسة الممتز المناعي المرتبط بالإنزيم (إليزا) قد يكون اختباراً مفيداً في تحديد أسباب الأمراض في سرطانات الرأس والعنق، ولكن النتائج غير قاطعة.

ABSTRACT We compared 50 patients with head and neck squamous cell carcinomas (cases) and 45 matched healthy controls. Biopsy specimens were taken from tumours and normal tissue of the cases and controls respectively and serial paraffin embedded sections were processed to detect Epstein–Barr (EB) viral antigen. We found EB viral proteins in 38% of cases and none in controls, which suggests a positive correlation. Serum samples were also tested for the presence of EB virus IgG by ELISA for comparison with immunohistochemical findings. Patients with positive immunohistochemical staining results had significantly higher mean antibody titres compared with those with negative results. ELISA may be useful in determining the etiology of head and neck cancers, but the results are not unequivocally reliable.

Le virus Epstein-Barr dans le cancer épidermoïde de la tête et du cou

RESUME Nous avons comparé 50 patients atteints de carcinome épidermoïde de la tête et du cou (cas) et 45 témoins appariés en bonne santé. Des prélèvements biopsiques ont été effectués sur des tumeurs et du tissu normal des cas et des témoins respectivement, et des coupes sériées incluses dans la paraffine ont été traitées pour détecter un antigène du virus Epstein-Barr (EB). Nous avons trouvé des protéines du virus EB chez 38 % des patients et aucune chez les témoins, ce qui indique une corrélation positive. Nous avons également recherché dans les échantillons de sérum la présence d'IgG anti-virus EB par ELISA pour comparaison avec les résultats immunohistochimiques. Les patients dont les résultats de coloration immunohistochimique étaient positifs présentaient des titres d'anticorps moyens significativement plus élevés par rapport aux patients dont les résultats étaient négatifs. Le test ELISA peut être utile pour déterminer l'étiologie des cancers de la tête et du cou, mais les résultats ne sont pas d'une fiabilité sans équivoque.

[1]Department of Microbiology, High Institute of Public Health; [2]Faculty of Dentistry, University of Alexandria, Alexandria, Egypt.
Received: 17/03/2002; accepted: 27/06/2002

Introduction

Head and neck cancer accounts for 5% of all malignant tumours, of which 95% are squamous cell carcinomas. Epstein–Barr virus (EBV) associated tumours tend to occur in the head and neck region, as the oropharyngeal epithelium is the main site of EBV proliferation after infection. EBV has been associated with a wide spectrum of malignant disease.

Patients with cancer often feel as though they have been invaded by an external force, yet the malignancies have arisen from their own tissues by a series of genetic alterations [*1*]. It is generally accepted that cancers have a multifactorial etiology with environmental and/or genetic risk factors playing a part. Increasingly, there is evidence that cancer may have an infectious etiology at least in part or in some cases [*2*].

Viruses are at the forefront of a large effort devoted to understanding the mechanisms of tumourigenesis-discovering how normal cells are transformed into cancer cells [*3,4*]. Both RNA-containing and DNA-containing viruses can cause various types of neoplasm in animals and humans [*5*]. The oncogenic DNA viruses are classified within several groups that include some herpes viruses [*4*]. EBV, a ubiquitous human herpes virus, is a member of the Gammaherpesvirinae subfamily. Antibodies to certain EBV-specific antigens are detectable in a large percentage of the general population [*6*]. The virus is commonly transmitted by infected saliva. Airborne or bloodborne transmissions are not important routes of infection [*7*]. EBV primary infection is usually asymptomatic or causes infectious mononucleosis [*5,8*]. EBV infects and replicates in oropharyngeal epithelial cells with the shedding of virus particles into the saliva and then infects B-cells where it persists in a latent state [*9*].

EBV has been linked to several malignant diseases, the most common of which are Burkitt lymphoma and nasopharyngeal carcinoma [*5*]. It has also been associated with other types of malignancies including Hodgkin disease, T-cell lymphomas, B-cell lymphomas, leiomyosarcoma and oral hairy leukoplakia in AIDS patients [*10,11*]. Several studies have provided evidence of the role of EBV as an etiologic agent of cervical cancer and breast cancer [*12–14*]. It has also been detected in other epithelial cancers in the head and neck region including carcinoma of the palatine tonsil, supraglottic laryngeal carcinoma and salivary gland cancer [*15*].

The diagnosis of EBV infection in head and neck cancers is based on antigen demonstration in tissue biopsies using several methods including immunohistochemical staining [*16*]. Serologic assays may aid in early diagnosis of occult cancers [*6*].

The aim of our investigation was to detect EBV in head and neck squamous cell carcinomas by studying the presence of EBV antibodies in serum and titres; by demonstrating the presence of EBV antigen in tissue biopsy specimens; and by comparing the significance of EBV antibody titre in serum with EBV antigen detection in tissue biopsy specimens.

Methods

We studied 50 cases with head and neck squamous cell carcinomas and 45 normal healthy individuals, matching the control group to the cases for age and sex. Biopsy specimens were taken from all cancer cases under general anaesthesia. Controls were patients admitted to the Departments of Maxillofacial Surgery and Periodontology for correction and restoration of mandible/maxilla traumatic fractures,

craniofacial plastic surgery, or treatment of tempromandibular joint ankylosis.

Biopsies of normal tissue were taken from the safety margin of the tumour tissue for the cases and from the control group matching for site, age and sex. Half of each tissue section from each case was formalin fixed, paraffin embedded and stained with haematoxylin and eosin to be screened by means of light microscope for diagnosis. Serial paraffin sections were processed to detect the EB viral antigen using the labelled streptavidin-biotin immunoperoxidase staining method (LAB Vision, Fremont, California, United States of America).

For serological study, venous blood (5 mL) was collected from each case and control. All serum samples were tested for the detection of EBV IgG by enzyme-linked immunosorbent assay (ELISA) (Meridian Diagnostic Incorporated, Cincinatti, Ohio, USA) to compare the results with the immunohistochemical findings.

Consent was taken from all controls before procedures.

Results

Of the 50 cases of squamous cell carcinoma, the immunohistochemical staining results for 19 (38%) were immunoreactive. Anti-EBV staining in all of the normal control sections was negative. EBV IgG was detected in 41 (82%) of the 50 cases of squamous cell carcinoma and in 42 (93.3%) of the 45 normal controls using ELISA. The EBV mean antibody titre in patients with head and neck squamous cell carcinoma was slightly elevated when compared with healthy controls although the difference was not statistically significant ($P = 0.2115$) (Table 1).

There was no statistically significant association between EBV antibody titre and age (Table 1) or sex (Table 2) of head and neck squamous cell carcinoma patients ($P > 0.05$). Comparing the ELISA results with the immunohistochemical staining results, we found that the sensitivity of the ELISA was 100%, the specificity 29%, the positive predictive value 46.3%, the negative predictive value 100% and the accuracy 56% (Table 3).

The mean antibody titre was significantly higher in patients with positive EBV immunohistochemical staining results than in those with negative results (Table 4).

Poorly differentiated tumours had the highest mean antibody titre (Table 5). Nevertheless, there was no significant association between the grade of the tumour and EBV antibody titre ($P = 0.7139$).

In terms of tumour site, patients with parotid tumours had the highest mean antibody titre, followed by those with tumours on the sinonasal area, the tongue, the buccal mucosa and the alveolar mucosa (Table 6). However, the association between the site of the tumour and antibody titre was not significantly associated ($P = 0.5782$).

Discussion

In our study, 19 of the 50 (38%) patients with squamous cell carcinoma were positive for anti-EBV antibody using labelled streptavidin-biotin immunoperoxidase staining. This was similar to an earlier study that found that 35% of squamous cell carcinoma cases were infected with EBV [*17*]. Similarly, EBV DNA has been reported in 33.3% of invasive squamous cell carcinomas as well as a positivity rate higher in malignant lesions than in benign ones, suggesting that EBV has a role in carcinogenesis [*18*]. However, sometimes it may exist in cancer cells as a passenger. Horiuchi et al. [*18*], Van Rensburg et al. [*19*] and Cruzet al. [*20*] noted a relatively

Table 1 **Comparison of Epstein–Barr virus antibody titres of head and neck squamous cell carcinoma cases and controls by age**

Age group (years)	Total		Negative ELISA		Positive ELISA							P-value[a]
			< 2		≥ 2		≥ 8		≥ 32			
	No.	%	No.	%	No.	%	No.	%	No.	%	Mean ± s	
< 20												
Cases	2	4.0	0	0.0	0	0.0	1	50.0	1	50.0	52.5 ± 53.0	Not
Control	1	2.2	0	0.0	0	0.0	1	100.0	0	0.0	19.0 ± 00.0	valid
20–39												
Cases	15	30.0	3	20.0	1	6.7	9	60.0	2	13.3	19.1 ± 25.8	0.5710
Control	14	31.1	0	0.0	4	28.6	7	50.0	3	21.4	24.6 ± 25.3	
40–59												
Cases	22	44.0	4	18.2	7	31.8	4	18.2	7	31.8	30.3 ± 36.6	0.9660
Control	23	51.1	3	13.0	2	8.6	11	47.8	7	30.4	30.7 ± 29.4	
≥ 60												
Cases	11	22.0	2	18.2	3	27.3	1	9.1	5	45.5	35.9 ± 37.1	0.4390
Control	7	15.6	0	0.0	1	14.3	5	71.4	1	14.3	23.6 ± 21.2	
Total												
Cases	50	100.0	9	18.0	11	22.0	15	30.0	15	30.0	29.1 ± 34.1	0.2115
Control	45	100.0	3	6.7	7	15.6	24	53.3	11	24.4	27.5 ± 26.3	

[a] *Mann–Whitney test.*
For cases: $\chi^2_9 = 14.753$, P = 0.098; *for controls:* $\chi^2_9 = 7.181$, P = 0.618.
s = *standard deviation.*

higher percentage using polymerase chain reaction (PCR), observing EBV in 100% of oral squamous cell carcinomas; the difference was most likely a reflection of the high sensitivity of PCR.

In contrast to our study, D'Costa et al. detected EBV in only 25% of oral squamous cell carcinomas in a group of Indian patients [*21*]. These differences in EBV prevalence may reflect inherent variations in the clinicopathology and molecular pathology of Indian oral cancer associated with the habit of chewing tobacco as compared with oral cancer associated with tobacco smoking and alcohol habits in other geographical areas.

Comparing ELISA and immunohistochemical staining results in our study, 19 (38%) of the patients were immunoreactive using the immunohistochemical staining method and 41 (82%) were positive using ELISA. Anti-EBV staining in the control sections revealed negative results, whereas 42 (93.3%) of controls were positive with ELISA. ELISA thus gave more false positive results. In other words, not every case testing positive for EBV antibody was accompanied by a positive result for EBV antigen in tumour tissues.

Similarly, Kottaridis et al. found that patients with nasopharyngeal carcinoma, patients with other carcinomas and cont-

Table 2 **Comparison of Epstein–Barr virus antibody titres of head and neck squamous cell carcinoma cases and controls by sex**

Sex	Total		Negative ELISA		Positive ELISA							*P*-value[a]
			< 2		≥ 2		≥ 8		≥ 32			
	No.	%	No.	%	No.	%	No.	%	No.	%	Mean ± *s*	
Male												
Cases	28	56.0	5	17.9	6	21.4	9	32.1	8	28.6	28.4 ± 34.1	0.984
Control	25	55.6	2	8.0	6	24.0	10	40.0	7	28.0	28.5 ± 29.1	
Female												
Cases	22	44.0	4	18.2	5	22.7	6	27.3	7	31.8	30.0 ± 34.9	0.676
Control	20	44.4	1	5.0	1	5.0	14	70.0	4	20.0	26.1 ± 23.0	
Total												
Cases	50	100	9	18.0	11	22.0	15	30.0	15	30.0	29.1 ± 34.1	
Control	45	100	3	6.7	7	15.6	24	53.3	11	24.4	27.5 ± 26.3	

[a]*Mann–Whitney test.*
For cases: $\chi^2_3 = 0.151$, P = *0.9851; for controls:* $\chi^2_3 = 4.89$, P = *0.179.*
s = *standard deviation.*

rols were 91.3%, 57.7% and 35.5%–69.2% sera positive for anti-viral capsid antigen (anti-VCA) respectively [22]. Sera positivity for anti-VCA was significantly higher in patients with nasopharyngeal carcinoma but was not significant in patients with other carcinomas. Antibodies to EBV observed in nasopharyngeal carcinoma and other neoplastic conditions could be accepted as evidence supporting the role of EBV infection in the etiology of cancer, but did not necessarily implicate EBV as an etiologic agent in human neoplasms.

Linde reported that 98% of healthy Swedish blood donors were EBV seropositive, adding that the specificity of the ELISA in detecting IgG anti-EBV antibodies for monitoring of nasopharyngeal carcinoma was not 100% [*23*]. This might be explained by the fact that EBV is a ubiquitous human herpes virus that persists for life following childhood infection.

Table 3 **Immunohistochemistry for Epstein–Barr virus antigen detection and ELISA for Epstein–Barr virus antibody detection for head and neck squamous cell carcinoma cases**

ELISA	Immunohistochemical staining					
	Positive		Negative		Total	
	No.	%	No.	%	No.	%
Positive	19	46.3	22	53.7	41	82
Negative	0	0.0	9	100.0	9	18
Total	19	38.0	31	62.0	50	100

ELISA sensitivity = 100%; specificity = 29%; positive predictive value = 46%; negative predictive value = 100%; accuracy = 56%.

Conclusion

Antibodies to certain EBV-antigens can be detected in a large percentage of the general

Table 4 **Comparison of the 41 positive ELISA for Epstein–Barr virus antibody titre detection for head and neck squamous cell carcinoma cases and their immunohistochemistry stains for Epstein-Barr virus antigen detection**

Immunohisto-chemical staining	ELISA positive cases (n = 41)							Total		P-value[a]
	≥ 2		≥ 8		≥ 32					
	No.	%	No.	%	No.	%	Mean ± s	No.	%	
Positive	3	15.8	6	31.6	10	52.6	47.2 ± 36.6	19	46.3	0.0359
Negative	8	36.4	9	40.9	5	22.7	24.7 ± 30.6	22	53.7	
Total	11	26.8	15	36.6	15	36.6		41	100.0	

[a] *Mann-Whitney test.*
s = *standard deviation.*

Table 5 **Comparison of ELISA and immunohistochemical staining test results with head and neck squamous cell carcinoma cases by grade**

Grade of squamous cell carcinoma	EBV positive[a]		Negative ELISA		Positive ELISA						
			< 2		≥ 2		≥ 8		≥ 32		
	No.	%	No.	%	No.	%	No.	%	No.	%	Mean ± s
Well differentiated (n = 24)	8	33.3	3	12.5	6	25.0	8	33.3	7	29.2	28.1 ± 33.9
Moderately differentiated (n = 19)	7	36.8	4	21.1	4	21.1	7	36.8	4	21.1	22.8 ± 28.7
Poorly differentiated (n = 7)	4	57.1	2	28.6	1	14.3	0	0.0	4	57.1	49.4 ± 45.0
Total (n = 50)	19	38.0	9	18.0	11	22.0	15	30.0	15	30.0	29.1 ± 34.1

[a] *By immunohistochemical staining.*
Kruskal–Wallis test, P-*value = 0.7139.*
χ^2_2 = *1.32,* P = *0.5165.*
s = *standard deviation.*

population. The detection of these antibodies indicates antigenic stimulation with the virus, although their presence in suspected cases cannot be used definitively for the diagnosis of occult head and neck cancers.

We found significantly higher mean antibody titres in patients with positive EBV immunohistochemical staining compared with those with negative results. This suggests that serologic assay (ELISA) may be useful in confirming the etiologic agent of head and neck cancers but it is not definitive. Demonstration of antigen in tissue biopsies remains essential for confirmation of the diagnosis.

That viral proteins were present in 38% of head and neck squamous cell carcinoma tissues and in none of the control specimens suggests a direct relationship between EBV and this type of cancer.

Table 6 **ELISA and immunohistochemical staining results of head and neck squamous cell carcinoma cases by tumour site**

Tumour site	Total (n = 50)		EBV positive[a]		Negative ELISA		Positive ELISA						
					< 2		≥ 2		≥ 8		≥ 32		
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	Mean ± s
Tongue	11	22	7	63.6	0	0.0	0	0.0	7	63.6	4	36.4	36.9 ± 35.0
Retromolar area	8	16	0	0.0	0	0.0	4	50.0	2	25.0	2	25.0	29.3 ± 38.1
Lip	6	12	3	50.0	1	16.7	3	50.0	0	0.0	2	33.3	29.2 ± 39.9
Palate	6	12	1	16.7	3	50.0	1	16.7	1	16.7	1	16.7	17.5 ± 35.6
Alveolar mucosa	5	10	3	60.0	1	20.0	0	0.0	2	40.0	2	40.0	35.2 ± 32.4
Floor of mouth	5	10	0	0.0	3	60.0	0	0.0	2	40.0	0	0.0	6.4 ± 07.5
Buccal mucosa	4	8	2	50.0	0	0.0	2	50.0	0	0.0	2	50.0	36.7 ± 38.5
Sinonasal area	2	4	1	50.0	0	0.0	1	50.0	0	0.0	1	50.0	36.3 ± 47.7
Larynx	2	4	1	50.0	1	50.0	0	0.0	1	50.0	0	0.0	8.3 ± 09.5
Parotid tumour	1	2	1	100.0	0	0.0	0	0.0	0	0.0	1	100.0	90.0 ± 00.0
Total	50	100	19	38.0	9	18.0	11	22.0	15	30.0	15	30.0	29.1 ± 34.1

[a]*By immunohistochemical staining*
Kruskal–Wallis test P-*value = 0.5782.*
χ^2_9 = *15.71,* P = *0.073.*
s = *standard deviation.*

References

1. Cavenee WK, White LR. The genetic basis of cancer. *Scientific American*, 1995, 272:72–9.
2. Cox MF, Scully C, Maitland N. Viruses in the aetiology of oral carcinoma? Examination of the evidence. *British journal of oral & maxillofacial surgery*, 1991, 29:381–7.
3. Poeschla EM, Wong-Stool F. Etiology of cancer: Viruses. In: Devita VT, Hellman S, Rosenberg SA, eds. *Cancer: principles and practice of oncology*, 5th ed. Philadelphia, Lippincott-Raven Publishers, 1997:153–84.
4. Joklik WK et al., eds. *Zinsser microbiology*, 19th ed. California, Appleton and Lange, 1988:729–56.
5. Brooks GF, Butel JS, Morse SA, eds. *Jawetz, Melnick and Adelberg's medical microbiology*, 21st ed. Norwalk, Connecticut, Appleton and Lange, 1998: 543–65.
6. Callaghan DJ, Conner BR, Strauss M. Epstein-Barr virus antibody titers in can-

cer of the head and neck. *Archives of otolaryngology*, 1983, 109:781–4.

7. Henle W, Henle G. Epstein-Barr virus and blood transfusions. In: Dodd RY, Barker LF, eds. *Infection, immunity and blood transfusion*. New York, Alan R. Liss, 1985:201–9.

8. Prang NS et al. Lytic replication of Epstein-Barr virus in the peripheral blood: analysis of viral gene expression in B lymphocytes during infectious mononucleosis and in the normal carrier state. *Blood*, 1997, 89:1665–77.

9. Grady JO et al. Epstein-Barr virus in Hodgkin's disease and site of origin of tumour. *Lancet*, 1994, 343:265–6.

10. Lam KY et al. Absence of Epstein-Barr virus in penile carcinoma. A study of 42 cases using in situ hybridization. *Cancer*, 1995, 76:658–60.

11. Thomas DW et al. Epstein-Barr virus in squamous cell carcinoma after renal transplantation. *Transplantation*, 1995, 60:390–2.

12. Labrecque LG et al. Epstein-Barr virus in epithelial cell tumors: a breast cancer study. *Advances in cancer research*, 1995; 55:39–45.

13. Se Thoe SY et al. Elevated secretory IgA antibodies to EBV and presence of EBV DNA and EBV receptors in patients with cervical carcinoma. *Gynecologic oncology*, 1993, 50:168–72.

14. Landers RJ et al. Epstein-Barr virus in normal, pre-malignant and malignant lesions of the uterine cervix. *Journal of clinical pathology*, 1993, 46:931–5.

15. Greenspan JS et al. Replication of Epstein-Barr virus within the epithelial cells of oral 'hairy' leukoplakia, an AIDS-associated lesion. *New England journal of medicine*, 1985, 313:1564–71.

16. Taylor CR. Immunoperoxidase techniques: practical and theoretical aspects. *Archives of pathology and laboratory medicine*, 1978, 102:113–21.

17. Mao EJ, Smith CJ. Detection of Epstein-Barr virus (EBV) DNA by the polymerase chain reaction (PCR) in oral smears from healthy individuals and patients with squamous cell carcinoma. *Journal of oral pathology & medicine*, 1993, 22: 12–7.

18. Horiuchi K et al. Epstein-Barr virus in the proliferative diseases of squamous epithelium in the oral cavity. *Oral surgery, oral medicine, and oral pathology*, 1995, 79:57–63.

19. Van Rensburg EJ et al. Detection of EBV DNA in oral squamous cell carcinomas in a black African population sample. *In vivo (Athens, Greece)*, 1995, 9:199–202.

20. Cruz I et al. Prevalence of Epstein-Barr virus in oral squamous cell carcinomas, pre-malignant lesions and normal mucosa — a study using the polymerase chain reaction. *European journal of cancer. Part B, Oral oncology*, 1997, 33: 182–8.

21. D'Costa J et al. Epstein-Barr virus in tobacco-induced oral cancers and oral lesions in patients from India. *Journal of oral pathology & medicine*, 1998, 27: 78–82.

22. Kottaridis SD et al. Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma and other neoplastic conditions. *Journal of the National Cancer Institute*, 1977, 59:89–91.

23. Linde A. Diagnosis of Epstein-Barr virus-related diseases. *Scandinavian journal of infectious diseases. Supplement*. 1996, 100:83–8.