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Serological profile of IGG antibodies to viral capsid antigen of epstein-barr virus in oral pre-cancer and cancer patients

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ABSTRACT

Introduction: Cancer of the oral cavity is a major and increasing public health problem. Oral cancer is the sixth most common cancer reported globally with an annual incidence of over 300,000 cases, of which 62% arise in developing countries. In India, because of cultural, ethnic, and geographic factors and the popularity of addictive habits, the frequency of oral cancer is high. Despite the various etiological and predisposing factors associated with oral pre-cancer and cancer, the role of co-carcinogenic effects of viruses like HSV has been documented. With this background and considering the high prevalence of oral cancer in India an attempt has been made to study the association of EBV with oral cancer using serological methods.

Aim and Objective: 1. To study the IgG antibodies to Viral Capsid Antigen (VCA) of Epstein-Barr virus in oral pre-cancer and cancer by a laboratory standardized Indirect Immuno Fluorescent (IIF) test. 2. To compare and corelate the results of IIF test with a commercially available ELISA for VCA IgG antibodies to EBV. 3. To assess the severity of disease progression in oral pre-cancer and cancer using the serological profile.

Results: In the present study, 84 subjects were assessed for serum IgG antibodies to VCA of EBV using the IIF technique and later the results were compared with an ELISA kit. Group I were controls, Group II to Group V were oral squamous cell carcinoma subjects according to the clinical staging. Group VI comprised of oral pre-cancer subjects. Age range was between 40-70 years, and the mean age was 50.31 +/- 8.23 years. All the 42 control sera were positive for IgG antibodies to VCA of EBV but with a significantly low geometric mean titre(GMT) of 8.561.The GMTs of oral squamous carcinoma and pre-cancer sera was positive but progressively increased when compared to control sera and were highly significant (P<0.001).

Conclusion: As there was a significant increase in the GMTs in oral squamous cell carcinoma subjects and pre-cancer subjects, serology of EBV may be interpreted to be useful in assessing the severity of the disease and the techniques of IIF and ELISA may be used as first line screeners when large number of subjects need to be assessed.

1. Introduction

Cancer of the oral cavity is a major and increasing public health problem in many countries both developed and non developed. It is the one of the 10 most common

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causes of death and oral cancer is the 6 most common malignancy in the world. Its incidence is significantly high were tobacco usage, betel nut chewing, beedi smoking and alcohol consumption are practiced. In India and many topical countries, most patients with this disease tend to seek medical consultation only at an advanced stage, thereby leading to poor disease monitoring and progress. The incidence of oral cancer is highest in India, South and South

east Asian countries. In India 90-95 % of the oral cancer is squamous cell carcinoma.

Despite the many causative and risk factors involved in oral cancer, in recent years, many attempts have been made to evaluate the potential role of viruses in oral cancers. The interaction of viruses with other carcinogens and oncogenes may be an important mechanism of disease.[1] Among viruses those associated with malignancies of the oral cavity include herpes simplex virus, human papilloma virus, human immuno deficiency virus, and Epstein - Barr virus. Techniques like polymerase chain reaction and DNA hybridisation, which are highly advanced have given substantial evidence proving the association of some of these viruses with Oral cancer.

A co carcinogenic effect between HSV and tobacco consumption has been demonstrated in animal studies.[2] Smokers demonstrate higher antibody titres to HSV suggesting reactivation[3,4] Epstein Barr Virus replicates within the upper spinous layer of epithelial cells in oral mucosa. Ultrastructural and virological study demonstrate a herpes group virus to be present in upper layer keratinocytes. Further, studies also reveal that EBV receptors have been found in normal and malignant oral epithelium.

With this background and considering the high prevalence of oral cancer in India a preliminary attempt has been made to study the association of EBV with oral cancer using serology.

2. Aims and Objective

1. To study the IgG antibodies to viral capsid antigen (VCA) of Epstein –Barr virus in oral cancer by a laboratory standardized Indirect Immunoflurescence test
2. To compare and co relate the results IIF test with a commercially available ELISA for VCA IgG antibodies to EBV.

3. Materials and Methods

The size of the study group was limited to forty two patients. The patients were selected from both sexes between age group of 40 -70 years. Only histopathologically proven cases of squamous cell carcinoma were selected the selected patients were introduced to the study before institution of any form of treatment, and none of them were suffering from any other systemic illness such as hypertension, diabetes mellitus and any infections. A brief case history was taken and appropriate clinical status of the patients was recorded as per the profoma. The control selected were normal individuals who were age and sex matched for oral habits.

3.1. Procedure

5 ml of blood was drawn from the anterior cubital vein of each individual aseptically using a pre-sterile plastic disposable syringe .The sample was transferred in to a 10 ml of centrifuge tube, kept at room temperature for at least 30 minutes and allowed to clot. Subsequently each sample was centrifuged and serum was obtained, the serum was then transferred to screw capped tubes stored at -20°C until further use.

3.2. Indirect immunofluorescence test for VCA antibodies to EBV

3.2.1. Principle of the test
When the test serum added to the same of the fixed cells expressing VCA antigen after washing, is treated with a fluorescent labelled antiserum to human gamma globulin, it reacts with the antibody molecules adherent to the antigen on the smear. After washing the unbound fluorescence conjugate, when viewed under the fluorescent microscope, apple green fluorescence is exhibited.Figure 1
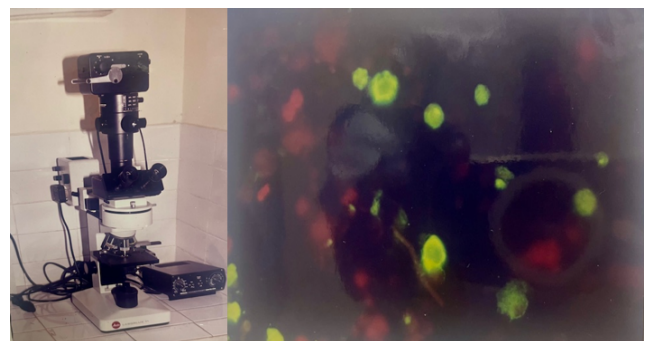
Figure 1: Apple green fluorescene under the fluorescent microscope

4. Viral Capsid Antigen Expression

An EBV producer cell line, P3HR1 which is lymphoblastoid in nature was used as a source of VCA. This cell line was obtained from the National Centre for Animal Tissue and Cell culture (NFATCC) Pune, P3HR1 was maintained in the laboratory by repeated passaging in RPMI 1640 supplemented with 20 % foetal calf serum at 37.C in 5 % CO2. At any given time 10 % to 15 % of the P3HR1 cells are known to express VCA (according to Werner Henle et al). VCA expression was induced in these cells by aging them at 32. C for 6- 9 days.

5. Procedure for Prepration of VCA

1. P3HR1 cells which had been aged at 32°c for 6- 9 days were removed by the low speed centrifugation. They were then washed with 2 % foet al calf serum in

phosphate buffered saline (0.1 M pH 7.5)
2. The cell concentration then adjusted to 10^6 viable cells /ml
3. One drop ($5x10^4$ cells) was added on to poly – 1 –lysine coated microscopic slides.
4. The smear were then allowed to air dry, and were then fixed in cold acetone for 10 minutes.
5. The fixed smear were then stored at -20^oC until for the use

Determination of the working dilution of the conjugate

The working dilution of the conjugate viz Fluoroescene isothiocyanate labelled antihuman gamma globulin was titrated in checker board reaction on aged p3HR1 cells and doubling dilutions of a known anti VCA antibody positive control serum. (kind gift from Dr. Hans Wolf, Germany) versus doubling dilutions of FITC conjugate. The smear were graded according to the intensity of fluorescence and the end point noted. The end point of the conjugate reactivity was considered as 1 unit the optimum dilution of the conjugate was taken as 8 units.

Titration of patients and control sera for IgG anti VCA antibodies.

The sera from both subjects and controls were tested by indirect immunofluorescence assay at doubling dilutions, starting with a dilution of 1:10 using smears. The reciprocal of the highest dilution of a serum giving visible fluorescence was considered as the titre of the particular serum sample.

Geometric mean titres (GMT) were calculated in each group. Statistical analysis was performed by analysis of variance based on log titres, by student t –test.

5.1. Enzyme linked immunoabsorbant assay (ELISA)

Detection of IgG antibodies to Epstein Barr Virus associated viral capsid antigen was done by using commercially available EBV (VCA) IgG ELISA kit (Virotech, system diagnostika, Germany)

Calculation of virotech units (VE)

This was as per the instruction of the manufacturer .Briefly , the virotech units of the cut –off controls has been defined as 10. The calculated VE of the positive controls had to be within the ranges mentioned in the quality control certificate. The extinctions of the bank values (450nm) were subtracted from all other extinctions.

VE(positive control) =OD positive control / OD cut off control x 10

VE (Patient serum) = OD patient serum / OD cut off control x 10

5.2. Interpretation of results

Samples with VE above 11 were regarded as positive. If the measured VE was with in the gray zone, no significant high antibody concentration was present.

VE	IgG Antibodies
<9.00	Negative
9.00 – 11.00	Gray Zone
>11.00	Positive

If the measured values were below the defined zone, no measurable antigen specific antibodies were present .

6. Results

In the present study, a total of 84 subjects were assessed for serum IgG antibodies to VCA of EBV using the IIF technique and later the results of IIF were compared by ELISA .The subject were divided in to 6 groups

1. Group I consist of 42 controls.
2. Group II comprised of 7 patients with stage II oral squamous cell carcinoma
3. Group III comprised of 7 patients with stage II oral squamous cell carcinoma
4. Group IV Comprised of 9 patients with stage III oral squamous cell carcinoma
5. Group V comprised of 14 patients with stage IV oral squamous cell carcinoma
6. Group VI comprised of 5 patients with oral precancerous lesions.

6.1. Age and sex

The study sample consisted of 42 patients out of these 42 patients 27 were females, and 15 were males (27:15).the age range was between 40-70 years .The mean age was 50.31+/- 8.23 years.

6.2. Site

With regard to the site of the lesions in the 37 cases of squamous cell carcinoma, 15 patients had involvement of the lower alveolus: 7 patients in the buccal mucosa, 4: patients – lower lip, 3 patients –lateral border of the tongue:3 patients hard palate: 2 patients floor of the mouth, 2 patients – retromolar trigone: 1 patient – oropharynx.

The precancerous lesion include 2 patients – Leukoplakia with severe dysplasia (bucccal mucosa)2 patients – leukoplakia with moderate dysplasia (buccal mucosa)1 patient carcinoma in situ (buccal mucosa)

6.3. Serum IgG level in controls

All the 42 control sera were positive for IgG antibodies to viral capsid antigen to EBV. Their serum IgG antibodies to viral capsid antigen of EBV . Their serum IgG titres from 5-20, the geometric mean titre (GMT) being 8.561

Table 1: Distribution of scc and precancerous lesions by site and sex

SL. No.	Site	Male	Female	Total
1	Floor of The Mouth	1	1	2
2	Oropharynx	-	1	1
3	Retromolar Trigon	-	2	2
4	Hard Palate	1	2	3
5	Tongue (V/L)	2	1	3
6	Buccal Mucosa	-	7	7
7	Lower Lip	-	4	4
8	Lower Alveolus	5	10	15
9	Leukoplakia(Mod.DYS)	1	1	2
10	Leukoplakia(Sev.DYS)	1	1	2
11	Carcinoma In-Situ	-	1	1
		11	31	42

6.4. Serum IgG level in subjects

All the sera from squamous cell carcinoma cases (37)and precancerous lesions (5) were positive for IgG antibodies to viral capsid antigen of EBV >The serum IgG level of test samples ranged from a titre of 20 -1280,with a geometric mean titre of 249.8.

The antibody titres in patients of stage I squamous cell carcinoma ranged from 20 -80 and the GMT was 44.16

In stage II the antibody titres ranged from 80-160 and the GMT was 195.0The antibody titre in stage III ranged from 160-320 and the GMT was 274.3

In stage IV the antibody titre ranged from 640-1280 and the GMT was 706.6

The serum IgG levels in precancerous lesions were also raised and the titre ranged from 80-640 with a GMT of 160.0 as seen in Table 2.

Table 2: IGG Anti-Vca Gmt in Oral Scc/Precancer cases and controls

Group	Stage	Geometric mean titre
I	Controls	8.561
II	Stage I SCC	44.16
III	Stage II SCC	195.00
IV	Stage III SCC	274.30
V	Stage IV SCC	706.60
VI	Precancerous Lesions	160.00

The GMT in all study groups were highly significant when compared to controls as seen in Table 3.

When a comparative study was made between the GMTs of Group II&III, II&IV,II &V,II&VI and Group III &IV, III&V, III &VI, and Group IV&V,IV & VI and Group V & VI the values were found to be significant as seen in Table 4.

Table 3: Correlation of GMT of controls and oral SCC cases

Groups	Controls	Cases	Statistical Sig.
Group I & II	42	07	P<0.001
Group I & III	42	07	P<0.001
Group I & IV	42	09	P<0.001
Group I & V	42	14	P<0.001
Group I & VI	42	05	P<0.001

Table 4: Comparison of 'p' value in controls and staging of SCC and precancerous lesions

Group	Statistical significance
I & II	
I & III	
I & IV	P<0.001
I & V	
I & VI	
II & III	P<0.001
II & IV	P<0.001
II & V	P<0.001
II & VI	P>0.500
III & IV	P>0.025
III & V	P<0.001
III & VI	P>0.500
IV & V	P<0.001
IV & VI	P<0.001
V & VI	P<0.001

7. Discussion

Understanding the etiology of a diseases is a critical step towards its control and prevention. Most diseases are known to have multiples etiology. One factor can usually be discerned as the single most important cause, but certain co –factors alo must be expressed for development of clinically apparent diseases.

In neoplastic diseases, mere exposure to an oncogenic agent (chemical or viral) is probably insufficient to induce cancer without the presence of other predisposing factors. This factor is of utmost significance when evidence for a viral association of cancer is evaluated .

The present study was undertaken to investigate the association of EBV with oral caner by studying the serological profile of IgG antibodies to VCA of EBV as a preliminary piece of work.

The patient who were introduced in to the study were thoroughly examined to rule out any systematic diseases by clinical examination, radiographs, routine blood and urine examinations. The patient with oral squamous cell carcinoma were staged depending on the

clinical presentation of the lesions, regional lymph node involvement and distant metastasis in to four stages following the TNM classification for cancer staging. Most of the patients in the study belonged to the lower socioeconomic strata who gave a history of profound smoking , tobacco chewing, and alcohol consumption .The age group of the individuals was between 40-70 years with mean age of 50.31years. The male to female ratio was 27:10. Most of the female patients gave a history of betel nut chewing or placement of tobacco quid in contact with the oral tissues.

IgG antibodies to viral capsid antigen of Epstein Barr virus in these patients was analysed by using Indirect Immunofluorescence (IIF) test. The results were at random correlated with that of a commercially available ELISA kit. (Virotech, Germany)

Comparison was made between the geometric mean titres of IgG antibodies to VCA of EBV in control groups and patients with oral squamous cell carcinoma. The GMT of controls who were age, sex, and also matched for habits was significantly low when compared to the GMT of oral squamous cell carcinoma patients.

These findings are in agreement with Coates et al.,[5] Pearson et al.[6] and Ringborg and co- workers[7] who found significantly high IgG antibody titres to VCA of EBV in oral cancer patients. Further the GMTs were found to progressively increase from stage I to stage IV, but were significantly higher in stage IV than in stage II and III.

The serum IgG levels in pre-cancerous lesions were also raised when compared to that of the control group. It was found that the GMT in all these study groups were highly significant when compared to the controls, which is in agreement with previous reports by Ghossein et al.[8] and Callaghan et al.[9] who found elevated IgG antibody titres to EBV anti-VCA in patients with oral cancer that increased with the stage of the disease.

Hence the serum IgG antibodies to VCA of EBV may be interpreted as being useful indicators in providing information regarding the severity of the disease.

Statistical significance was not observed between stage II and stage III disease which may perhaps be attributed to the degree of immunosuppression being less in stage II when compared to stage III disease. On the contrary, it has been reported by Henle at al[10] that nodal involvement may lead to enhanced antibody production.

However, the reason of elevated IgG anti –VCA titres in stage III diseases onwards may represent reactivation of latent EBV infection that occurs after a state of immunosuppression develops in the host from the effects of the tumour.

DNA studies however, suggests that EBV involvement in oral cavity carcinogenesis may follow the initial action of some other agent –infectious, chemical, or traumatic in facilitating EBV entry in to the epithelial cell.[11]

The rise in the antibody titre as the disease progressed may be attributed to the interaction of EBV with environmental co-carcinogenesis. However the fact whether EBV is a co –factor in oral carcinogenesis or is merely a passenger virus needs to be evaluated to associate EBV with oral cancer. This can be firmly established only after DNA studies with biopsy tissues has been carried out amongst these patients.

8. Conclusion

Evaluation of IgG antibodies to viral capsid antigen of Epstein- Barr virus in oral cancer was selected as the subject of this study. The emphasis was on the serological profile of IgG antibodies to VCA of EBV in different stages of oral squamous cell carcinoma, pre-cancerous lesion patients and age, sex matched healthy controls. We can state that serology of Epstein barr virus in oral cancer patients may be interpreted to be useful in assessing the severity of the disease .The techniques of indirect immunofluorescence, and ELISA may be useful as first line screeners when a large number of samples need to be assessed. However a definite conclusion between the association of EBV and oral cancer can be firmly established only after DNA studies have been done.

9. Source of Funding

10. Conflict of Interest

None.

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