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Association of Herpes Simplex Virus with Human Oral Carcinoma

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INTRODUCTION: Association of Herpes Simplex Virus (HSV) type 2 with human uterine cervical cancer has been firmly established(1,2). Oral cavity, as in the case of uterine cervix, is a major site or recurrent herpetic infection. Cancer of the mouth, as well as of the cervix, is predominantly squamous cell tumor. Patients with oral premalignant lesions were shown to be associated with cell-mediated immunity against HSV1. Oral cancer constitutes about 27% of the total cancer cause registered in this region(3). This is one of the highest prevalence rates seen anywhere in the world. Oral cancer has been shown to be associated with the habit of chewing tobacco, but other factors, including viruses as etiological agents, are to be carefully evaluated. Previous reports showing asociation of Herpes Virus with oral cancer are scanty. We had elsewhere reported that the antibodies against HSV1 are increased in oral cancer patients(4,5) and the oral cancer cells contain HSV related antigens on their surface(6). Here we extend the work and also show the presence of HSV1 genome in the oral cancer cells.

MATERIALS AND METHODS:

Antibodies in circulation: Presence of antibodies against HSV was determined by indirect Hemagglutination test (7) and confirmed by Hemagglutination inhibition test in most of the samples; micro neutralisation test (8) was also done, which confirmed the results. Since HSV infection is ubiquitous in this region, only those sera showing an antibody titre of 32 and above were taken as positive. The viral antigen was prepared by the method described previously (7).

Antigen on cell surface: Punch biopsy specimen from oral cancers were collected in medium, washed, cut into small

pieces, minced and made into single cell suspension. Cells were washed three times, finally resuspended in a few drops of PBS and smears were prepared, air-dried and fixed in acetone for 10 minutes. Direct and indirect immufluorescence techniques using specific HSV1 antiserum were employed.

DNA hybridisation studies: (a) DNA was extracted from homogenized specimen by phenol and chloroform-isoamylalcohol mixture and precipitated by ethyl alcohol. The DNA strands were disrupted by NaOH treatment at room temperature. The DNA was quantitated by optical density at 260 nm. The DNA was resuspended in Tris-EDTA solution, and 50 ul, containing 10 ug of DNA was added on Nitrocellulose membrane (Schliecher & Schuell, BA-85) applied on Hybri-Dot Manifold (BRL, Bethesda). A series of dots containing DNA was prepared on each filter. Each well was then washed twice, baked at 80° c for 2 hours, placed in sealed plastic bags containing prehybridization mixture with calf thymus DNA for 2 hours at 50°c.

(b) ECoRI fragments of HSV1 (D&I) were cloned in pGEM32 plasmid vector; ECoRI fragment (E + K) were cloned in pBR 325 plasmid and Bgl IIn fragment of HSV2 was cloned in pBR 322 plasmid vector. These along with the whole HSV1 genome were used as probes. These vectors were amplified in E Coli. The cloned HSV1 fragments were nick translated by employing DNA polymerase I and DNAase I and incubating with p32-dCTP at pH 7.5 for 60 min at 15° C. The reaction was stopped by sodium EDTA. Spun column chromatography was employed to separate labelled DNA from unlabelled nucleotides. Specific activity of about 10^7 cpm/ug was achieved.

(c) Prehybridised host cell DNA samples on nitrocellulose were then incubated with nich translated viral probe and hybridization was carried out at 50° C in thermally sealed plastic bags for 18 hours with gentle lshaking. Then filters were washed 6 times, air-dried and finally autoradiographed with Kodak XAR film in cassettes containing image intensifier screens for 6 hours to 48 hours at -70° C, and then developed in a Kokad X-omat processor for 4 hours.

(d) In situ hybridization: The above mentioned HSV1 fragments were used. Random priming method was employed for probe synthesis. The insert DNA of HSV1 was separated from the vectors and denatured at 100° C for 2 min, reacted with DNA polymerase I and S35-dCTP at RT overnight. Carrier DNA was

added to this mixture. DNA was precipitated by phenol-chloroform & finally ethanol precipitation was done. The glass slides were treated with 3-aminopropyl-triethoxy silane, washed further with gluteraldehyde, and washed finally with sodium periodate. Paraffin-embedded, cut sections of malignant and non-malignant tissues were mounted on slides, deparafinised and hydrated by passing through descending grades of ethanol and finally brought to phosphate buffered saline. The sections were treated with 0.2 N HCl for 20 min, followed by treatment with proteinase K for 15 min, washed, then acetylated with acetic anhydride in triethanolamine for 10 min, washed and finally dehydrated using ascending ethanol grades. Hybridization mixture containing DNA probes (about 10^6 cpm per 12 ul) were added on the tissue sections, covered with siliconized cover slips and sealed, incubated at 100° C for 10 min, then 0° C for 10 min, and finally at 25° C for 2 days. Later, cover slips were removed, washed and autoradiographed at 4° C for 6 days. The slides were then developed, fixed and then stained with hematoxylin-eosin. The developed grains on the cell nuclei were noted.

RESULTS: Prevalence of HSV1 antibodies in different groups, studied by indirect hemagglutination technique, is shown in Table 1. Out of 986 oral cancer patients, 601 (61%) showed the presence of HSV1 antibodies, as against 293 (39%) in 751 healthy control subjects (P 0.001). It may be argued that any virus could npn-specifically invade the cancer cells leading to these results. To rule out this possibility, as a negative control, antibodies against adenoviruses were used in 200 oral cancer cases and 151 controls. Both HSV1 and adenovirus infections are common in this region, as shown by the control values. But only HSV1 antibodies not the adenovirus antibodies did show any increase in the oral cancer groups compared (Table 2). Table 3 shows HSV1 antibody titres in cancer groups compared with normals. The percentage positivity at each titre is more in oral cancer patients, as compared to normal controls. Thus the anti-HSV antibodies are not only more prevalent in cancer group, but also that the patients had increased titre values. The tumour size has very little relationship with the percentage positivity of HSV antibodies in oral cancer patients (Table 4), showing that the antigenic stimuli are present in tumours of any size. The HSV1 antibodies in patients who came for follow up at regular intervals were studied. Among the 20 patients thus studied, 12/20 (60%) had decreasing titres during the follow up period. This shows that the stimulation to produce antiviral antibody was lost when the tumour is clinically removed. In certain patients, the antibody titre, was seen to be increased, may be the forerunner of the recurrence of the disease.

Table 5 shows the results of fluorescence studies, made on cancer tissues. HSV1 infected Vero cells served as positive controls, and non-infected Vero cells as negative controls. Among the 20 normal control specimens, only 30% showed fluorescence; but 135/175 (77%) cancer specimens showed brilliant fluorescence. The specificity of the reaction was confirmed by the loss of fluorescence when the antiserum was treated with HSV1 infected Vero cells. Anti HSV2 serum showed positive fluorescence in 30% specimens, which could be explained by the similarity between the 2 viral strains.

Results of the immunoperoxidate staining are presented in Table 6. 124/175 (71%) of the oral cancer specimens were positive, while only 4/20 (20%) of the normal control specimens showed positivity.

Since the HSV1 antibodies were more in oral cancer patients, and the HSV1 antigens were on the oral cancer cell surface, the next logical step was to test whether the HSV genome was present in oral cancer cells. For this, the DNA from cancer specimens were extracted, denatured, precipitated on nitrocellulose membrane and hybridized with nicktranslated cloned HSV probes. The results are given in Table 7. It is seen that whole HPV16 probe was hybridized with 10% oral cancer and 37% of cervical cancer specimens. The normal cells did not hybridize with viral probes. The HSV and HPV genes are generally mutually exclusive, as very few samples contain both viral genes concurrently.

In situ hybridization studies using ECoRI D&I fragments of HSV1 showed 60% (9/15) positivity in oral cancer specimens (Table 8) 12% (1/8) of the non-malignant, control tissue specimens also showed positivity.

DISCUSSION: The above experiments strongly shows the relationship of HSV with oral cancer. The HSV1 antibodies are consistently higher in oral cancer patients, the HSV1 related antigens are shown to be on the oral cancer cells and the HSV probe could be hybridized with the DNA from oral cancer specimens. Even though there are scanty reports (9,10) on the serological relationship of HSV with oral cancer, ours is the first attempt to demonstrate the relationship by using such large number of serum samples. Further, we could extend these results by immunofluorescence and DNA hybridization techniques. From the studies reported here, we could say that the HSV1 genes are related with oral cancer. Whether the viral gene is integrated could be established only by restriction analysis of DNA, which we are now

attempting; this study is in progress. Epidemiological evidence indicate that tobacco chewing and smoking are major risk factors in oral cancer(11). In view of the present study, it can be assumed that the HSV may play a role as promoter or co-factor in the etiology of oral squamous cell cancers.

TABLE - I

SERUM ANTIBODIES AGAINST HERPES SIMPLEX VIRUS IN CANCER PATIENTS

Groups Studied	Total No. of Sera	No. of Sera Positive	%+ve	P.Value
Oral Cancer	986	601	61	0.001
Uterine cervix cancer	300	138	46	-
Normal controls	751	293	39	-

TABLE - II

SERUM ANTIBODIES AGAINST ADENOVIRUS IN CANCER PATIENTS

Groups Studied	Total No.	No.+ve	%+ve	P.Value
Oral Cancer	200	80	40	N.S.
Cervical Cancer	110	53	48	N.S.
Normal controls	151	71	47	N.S.

TABLE - III

ANTIBODY TITRES AGAINST HERPES SIMPLEX VIRUS

Groups Studied	Total No.		Titre Value				
			32	64	128	256	512
Oral Cancer	608	No + ve	80	86	84	60	50
		% + ve	13.2	14.1	13.8	9.9	8.2
Normal Controls	151	No + ve	6	16	19	12	7
		% + ve	3.9	10.5	12.6	7.9	4.6

TABLE - IV

STAGE OF CARCINOMA WITH VARIATION IN HSV1 ANTIBODY DISTRIBUTION

Clinical stage	Total No. Tested	No. +ve	% +ve
T_1	6	2	-
T_2	111	57	51.4
T_3	126	82	65.1
T_4	131	76	58.0

TABLE - V

DETECTION OF HSV1 ANTIGEN ON CELL SURFACE BY IMMUNOFLUORESCENCE METHOD

Specimen from	No. Tested	No. +ve	Percentage of Positivity
Normal Oral	20	6	30%
Oral Cancer	175	135	77%

TABLE - VI

DETECTION OF HSV1 ANTIGEN ON CELL SURFACE BY IMMUNOPEROXIDASE TECHNIQUE

Specimen from	No. Tested	No. +ve	% of Positivity
Normal Oral	20	4	20%
Oral Cancer	175	124	71%

TABLE - VII

SLOT BLOT TECHNIQUE OF HYBRIDIZATIONS OF DNA FROM CANCER SPECIMENS WITH NICK TRANSLATED PROBES

Probe	Non Malignant	Oral Cancer	Cervical Cancer
HSV_1	4/23 = 20%	41/71 = 58%	
HSV_1 EK	1/11 = 10%	9/21 = 43%	
HSV_1 D&I		2/20 = 10%	
HSV_2	1/12 = 10%		38/102 = 38%
HPV_{16}	0/12 = 0%	5/56 = 10%	25/69 = 37%
HSV + HPV	0/12 = 0%	3/50 = 6%	4/102 = 4%

TABLE - VIII

IN SITU HYBRIDIZATION USING TISSUE SECTIONS AND ^{35}s-VIRAL PROBES

	Non-Malignant	Oral Cancer	C.Can
HSV_1 -ECoRI-D & I	1/8 = 12%	9/15 = 60%	
HSV_2 -Bg_1 II-N	0/1 = 0%	0/2 = 0%	
HPV_{16} BamH	2/8 = 25%	2/10 = 20%	7/11=64%

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