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Role of Herpes Simplex Virus Type-2 in Carcinoma of the Human Uterine Cervix

V. Thankamani* and D. M. Vasudevan**

*Department of Microbiology, College of Engineering, Trivandrum-14 **Amla Cancer Research Centre, Trichur-53.

ABSTRACT

The role of Herpes simplex virus type-2 in the aetiology of carcinoma of the uterine cervix was investigated. Sera from 400 cervical cancer cases, 250 healthy age matched controls and 890 other cancers were tested for the presence of HSV-2 specific antibodies to whole virion antigens by neutralization and indirect haemagglutination tests. A statistically significant high percentage of cervical cancer patients had higher titres of HSV-2 antibodies compared to controls. Among the controls the increase in HSV-2 antibodies was significantly age dependent whereas it was completely independent of the age factor in cancer cases. By indirect immofluorescent technique 169/215 cervical cancer biopsies showed the presence of Herpes simplex virus type-2 antigens. The results of serological studies, tissue screening and in-vitro tests of cell-mediated immunity to HSV-2 were indicative of definite role of this virus in cervical cancer. By peroxidase-antiperoxidase technique 28/64 of cervical tumor tissues revealed the presence of Human papilloma virus antigens in the nuclei of the tumour cells. Further work is being carried out to elucidate the role of these two viruses, HSV-2 and Human papilloma virus, proved to cause natural tumours in animals and man respectively, in the genesis of uterine cervical cancer. At this juncture attempts have been made to develop cell limes from tumour tissues to test the cytotoxic effect of interferon against malignant cells envisaging future application of interferon either alone or in combination with other drugs as a valuable tool to achieve complete regression of the tumour especially in advanced stages like stage II and III following conventional radio therapy—which at present has secured only incomplete regression.

Herpes viruses have been associated with neoplasia in humans and animals. Evidences in favour of a close association between Herpes simplex viruses Type-2 and neoplasia have mounted over the last two decades. Analysis of the data collected by the Tumour Registry, ICMR, at the Regional Cancer Centre, Trivandrum, during the year 1982-83 revealed that out of the

3483 new cases recorded, 1629 (46.7%) were females and 1854 (53.2%) males. Oral cancer accounted for 24.6% of the cases followed by cancer of the uterine cervix (12.3%) in the total sample. Among the females subjects, however, certical cancer ranked first (26.4%) as the most frequent site of cancer followed by the oral cancer (17%).

The high rate of incidence of cervical cancer in this area prompted a detailed study of the possible aetiological role of Herpes simplex virus and human papilloma virus in the genesis of squamous cell carcinoma of the uterine cervix.

Biopsies from clinically diagnosed and histopathologically proved cervical cancer cases and blood samples were collected from patients attending the radiotherapy clinic of the Regional Cancer Centre, Trivandrum; department of Gynaecology and Obstetrics, S. A. T. Hospital; department of Skin and Venereal Diseases; the Blood Bank and staff of the Medical College Hospital, Trivandrum. All sera were stored at-20°C.

The prevalence rate of Herpes smplex virus type-2 infection in the general population was assessed. Serum samples were tested for specific antibodies to HSV-2 antigens by the indirect haemagglutination test and complement fixation test using the II/I threshold index. Out of the 500 subjects tested 135 were considered positive since they had II/I index of over 85, giving an incidence of 27% positive response. Secondly blood samples from cervical cancer cases and various other control groups were collected and sera analyzed for specific antibodies to HSV-2 employing neutralization tests in Vero monolayers, and indirect haemagglutination inhibition tests with HSV-1 and HSV-2. Specific reactivity was determined by the II/I index. The results summarised in Table I shows that compared to normal healthy females, cervical cancer patients showed significantly higher percentage positivity with respect to HSV-2 antibodies, the p-value being <0.01. Cases of dysplasia and cancer-in-situ, which are considered to be preceding invasive carcinoma showed 58% positivity, (p-value >0.01). This suggests that women infected with HSV-2 are

TABLE 1. Prevalence of HSV-2 antibodies in various groups

Study group	No. of cases	No. positive	% positive	p-value
Normal	250	91	36	
Normal, pregnant	53	20	38	>0.05
Skin and V.D.	270	119	44	>0.05
Dysplasia, cancer-in situ	40	23	58	>0.01
Invasive carcin-oma cervix	400	282	70	<0.01

probably at a higher risk of developing cervical cancer than non-infected women.

It was observed that among control (normal) subjects, HSV-2 antibodies commence to appear at puberty thereafter rising steadily upto 40 years and then fall to lower levels. On the other hand, in cervical cancer patients, irrespective of the age, the antibody titres to HSV-2 antigens and percentage positivity were quite high and uniform. The number of normal females having high antibody titres were much lower (38%) in comparison with the 78% positivity in cervical cancer patients with very high titres from 64 to 512 and above.

The findings of the serological tests were further confirmed by indirect immunofluorescent technique. Vero monolayers infected with HSV-2 were smeared on slides, air dried and fixed in cold acetone. They were covered with cervical cancer patients' sera, incubated and followed by FITC conjugated anti-human immunoglobulin. Sera from 50 patients of cervical cancer were tested and 42 reacted with Vero cells infected with HSV-2 (24-48 hrs) giving a positive immunofluorescent response rate of 84%.

In order to look for the presence of any HSV-2 specific virus antigens in the tumour tissues, the biopsies from clinically diagnosed and histopathologically proved cases of squamous cell cervical carcinoma; tissue specimens from normal cervix from operation material, cancer breast etc. were gently minced and single cell suspensions were prepared. The cells were washed, pelletted and smears prepared. They were fixed in cold acetone and preserved at -20°C. Formalin fixed paraffin sections 5-10 μ thick were also used. All control tissues too were prepared identically. Parallel sections and smears were stained by routine haemotoxylin and eosin to localise the tumour cells and confirm their malignant characteristics cytologically. Parallel specimens were tested for HSV-2 antigens by indirect immunofluorescence and HRPO-conjugated HSV-2 antibody staining. The results are shown in Table 2. Appropriate reagent controls were included in every test series.

TABLE 2. HSV-2 antibodies in tissues from carcinoma cervix by indirect immunofluorescence and HRPO HSV-2 antibody straining.

Test	No. tested	No. positive	%
Indirect immunofluorescence (Rabbit HSV-2 Ab, FITC antirabbit Ig)	215	169	79
HRPO-HSV-2 antibody	50	41	82

The fluorescence was predominantly nuclear though membrane and cytoplasmic fluorescence were also noted. This provided supportive data for the expression of HSV-antigens on tumour cells. Twenty three out of 40 cases

(58%) of dysplasia and cancer-in-situ were positive. Probably HSV-2 acts as an inducer in the process of carcinogenesis. The 23% positivity in PBS substituted for HSV-2 antibody reflects the nonspecific Igs present on the immunoactive cells in the tumour mass or the HSV-2 antibodies deposited on the tumour cells acting as blocking antibodies. The membrane fluorescence or HRPO staining could be reasoned out by the several structural modifications brought about by previous infection with HSV-2 and the incorporation of virus coded proteins in the nuclear and cell membranes.

Attempts were made to isolate infectious virus by in vitro culturing of tumour cells and co-culturing with Vero cells. None of the specimens revealed any virus specific cytopathic effects out of the 50 samples cultured. This suggests that transformation does not necessitate the complete viral genome but rather results from the expression of specific viral gene(s). Hence, whatever the stress, infectious virus cannot be induced at least in most of the transformed cells.

The next series of tests were aimed at detecting tumour associated viral antigens on the cancer cells, using autologous patients' sera by indirect immunofluorescent technique. More than 85% of samples expressed brilliant fluorescence, indicating the presence of antibodies in patients' sera against the tumour specific antigens on the tumour cells. Further each serum was absorbed with HEP-2 cells infected with HSV-2 and the test repeated on serial sections of the same samples. The fluorescence, interestingly enough, was reduced to 40%. It is possible therefore that tumour tissue antigens were HSV induced. Exhaustive absorption might have produced even lesser % positivity.

Further, patients' sera were tested for the presence of antibodies against AG-4, an early virus induced protein synthesised in HSV-2 infected HEP-2 cells between 4-6 hrs after adsorption and a positive response was obtained in 72% patients of carcinoma cervix (Table 3). A positive response was also

TABLE 3. Serum antibodies to AG-4

Sample	No. of cases	No. positive	% Positive
Carcinoma cervix sera	25	19	72
Normal sera	10	0	0
Breast cancer	6	0	0
Lymphoma	5	0	0
Leukemia	5	0	0
Dysplasia, cancer-in-situ	10	6	60

obtained from the serum samples of cases of dysplasia and cancer-in-situ but in a somewhat smaller % of cases. The results were negative in the serum samples of the other groups of subjects, as indicated in Table.

To obtain further confirmation of the results obtained with the serum samples cervical tumour biopsies were formalin fixed and paraffin sections prepared. These, along with necessary known (+)ve and negative tissues, were analyzed with peroxidase conjugated anti-HPV genus specific structural antigens by PAP technique (Supplied by Dakopatts). Their presence could be demonstrated in a high percentage of tissues from the cases of cancer cervix and in none of the cases of normal cervical tissue (Table 4).

TABLE 4. Presence of HPV genus antigens in cervical tissue from normal and cancer sites.

Group	PAP No.	Cancer cervix No. positive	% Positive
Cancer cervix	64	28	43
Normal cervix	4	0	0

Next to Herpes simplex virus type-2, human papilloma virus has been incriminated as the most probable candidate among the possible aetiological micro-organisms in the genesis of cervical cancer.

DNA extracts from 102 samples of cervical cancer tissues analyzed by molecular DNA-DNA hybridization techniques with HSV-2 DNA and HPV-DNA probes brought out very exciting results. Fifty two percent samples revealed Herpes simplex virus type-2 DNA sequence and 30% contained human papilloma virus DNA sequence . Futther work is in progress.

The results of these investigations provide a strong basis for suggesting definite association of Herpes simplex virus type-2 and/or human papilloma virus in the presence of other obscure factors in the genesis of squamous cell carcinoma of the human uterine cervix.

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