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Detection of HSV_1 DNA segments in human oral cancer biopsies by dot-blot and in-situ DNA hybridisation techniques

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Radiolabelled HSV_1 probes were seen to be hybridised with the DNA isolated from oral cancer tissues when studied using dot-blot and in-situ hybridisation techniques. The HSV_1 middle segment (M-A fragment) probe hybridised with the DNA from 51 out of 80 (64%) oral cancer tissues, as against 5 out of 25 (20%) normal tissues. HSV_1 E+K fragment hybridised with the DNA from 45 out of 80 (56%) oral cancer tissues. Only 8% (2 out of 25) DNA samples from normal tissues showed hybridisation with this fragment. In-situ hybridisation studies, using ECoRI D and I fragments of HSV_1, showed 60% positivity (48 out of 80) in oral cancer tissues. Only 12% (3 out of 25) of the tissue samples from normal healthy subjects showed positivity, when studied with these probes. The results of the present study further confirm the association of HSV_1 with oral carcinoma, as was demonstrated by previous seroepidemiological and immunohistochemical studies by our group.

Key Words: Oral cancer, HSV_1, DNA hybridisation.

Viruses have been reported to be associated with many types of cancer (1, 8, 17). The association of HSV_2 and HPV with the carcinoma of uterine cervix is well-established (4, 14). Oral cavity, as in the case of uterine cervix, is the major site of recurrent Herpetic infection. Sabin (13) and Shillitoe et al. (15) have found high antibody titres against HSV in oral cancer patients. RNA complementary to HSV_1 was observed in human oral squamous cell carcinoma (5). HSV_1 related proteins were detected in oral cancer cells (7, 16). Seroepidemiological studies from our group have shown a high prevalence of HSV_1 specific antibodies in oral cancer patients (9, 10). The association of HSV_1 was further strengthened by demonstring HSV_1 related antigens on oral cancer cells by immunohistochemical methods in our earlier studies (11). In the present study, DNA hybridisation techniques have been used in an attempt to establish the association of HSV_1 with oral carcinoma by locating the viral DNA in the genome of oral cancer tissues.

Materials and Methods

Tissue samples from 80 histopathologically proved oral squamous cell cancer cases were collected for the study. Twenty-five normal tissue samples, collected from fresh

autopsy specimens, were used as control. DNA was collected from all these samples individually by the method described by Botchan et al. (2).

ECoRI fragments, HSV_1 D, HSV_1 I and HSV_1 M-A were cloned in PGEM 32 plasmid vector and the ECoRI fragment (E + K) was cloned in PBR 325 plasmid vector. These vectors were amplified in *E. Coli*. The cloned HSV_1 fragments were nick translated by employing DNA polymerase I and DNAase I and incubating with p ^{32}-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/μg was achieved. This was used for dot-blot hybridisation.

The HSV_1 fragments (D and I) were used for in-situ hybridisation. Random priming method was employed for probe synthesis. The insert DNA of HSV_1 was separated from the vectors and denatured at 100° C for 2 min., reacted with DNA polymerase I and S ^{35}-dCTP at room temperature over-night. Carrier DNA was added to this mixture. DNA was precipitated by phenol-chloroform, and finally ethanol precipitation was done.

Dot-blot hybridisation studies were performed following Kafatos et al. (6). DNA was extracted from homogenized specimens by phenol and chloroform isoamyl alcohol mixture and precipitated by ethyl alcohol, and strands were disrupted by NaOH treatment. 50μl of Tris-EDTA solution, containing 10μg of DNA, was applied on nitrocellulose membrane (Schliecher and Schuell, BA-85) placed on Hybri-Dot Manifold (BRL, Bethesda). DNA from each sample was applied in different slots. A series of dots containing DNA was prepared. The blots were washed, baked at 80° C for 3 hrs., and prehybridised in sealed plastic bags containing calf thymus DNA for 2 hrs. at 50° C.

Prehybridised host cell DNA samples on nitrocellulose membranes were then incubated with 1 μg of nick translated viral probe and hybridization was carried out at 50° C in thermally sealed plastic bags for 18 hrs. with gentle shaking. The filters were then washed 6 times at high stringency conditions, air-dried and autoradiographed with Kodak film in cassettes containing image intensifier screens for 48 hrs. at –70° C, and then developed in a Kodak X-omat processor for 4 hrs. The unknown dots were graded as positive when film dot intensity was equal to or greater than standard dot for 50 μg of HSV DNA.

In-situ hybridisation was done following the method of Brahic and Haase (3). The glass slides were treated with 3-aminopropyl-triethoxy-silane, washed with glutaraldehyde, and sodium periodate. Paraffin-embedded, tissue sections (4-6 μm thick) of malignant and non-maligant tissues were mounted on slides, dewaxed, hydrated and brought to phosphate buffered saline. The sections were hydrolysed with 0.2 N HC1 for 20 min., then treated with Proteinase K for 15 min., washed, acetylated with acetic anhydride in triethanolamine for 10 min., washed and finally dehydrated using ascending grades of ethanol. Hybridisation mixture containing DNA probes (about 10^6 cpm per 12μl) were added on the tissue sections, covered with siliconized cover slips, sealed, incubated at 100° C for 10 min., then at 0° C for 10 min. and finally at 25° C for 2 days. After removing the cover slips, the sections were washed and autoradiographed at 4° C for 6 days. The slides were then developed, fixed and stained with hematoxylin-eosin. The developed grains on the cell nuclei were assessed.

Results and Discussion

The results of the dot-blot and in-situ hybridisation studies are presented in Table I. It is seen that 56-64% of oral cancer tissues were hybridised with HSV_1 probes, as against 8-20% positivity in normal controls.

There is a very high incidence of oral cancer in Kerala (12). Many etiological fac-

Table I - Results of dot-blot and in-situ DNA hybridisation studies using HSV_1 probes

Technique	Probe	Normal controls, positive	Oral cancer specimens, positive
Dot blot	HSV_1 M-A	5/25 (20%)	51/80 (64%)
do	HSV_1 E+K	2/25 (8%)	45/80 (56%)
do	HSV_1 D	2/25 (8%)	34/60 (57%)
In situ	HSV_1 D	3/25 (12%)	48/80 (60%)
do	HSV_1 I	3/25 (12%)	48/80 (60%)

tors, such as chewing of tobacco and/or smoking have been attributed to this high incidence. Our earlier studies have shown a close associationship of HSV_1 with oral cancer (9, 10, 11). Based on a large number of samples, we were the first to demonstrate the association of HSV_1 with oral cancer by seroepidemiological and immunohistochemical studies, where 60-70% positivity was observed. The present study demonstrates the presence of HSV_1 DNA in the DNA of a considerable number of tissue samples from oral cancer patients, and lend further support for the association of HSV_1 and oral cancer. At this stage we are unable to say whether or not the viral genes are incorporated into the genome of oral cancer cells. Further studies using restriction analysis of DNA are in progress to verify this probability.

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