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# Search for HSV DNA in Genital, Cerebral and Labial Tumors

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**Abstract**—*DNA sequences homologous to HSV-1 and HSV-2 DNA fragments were searched in 64 genital, 35 labial and 34 cerebral tumors. Southern blot transfers of tumor and control DNAs were hybridized in stringent conditions with $^{32}P$ labelled probes from HSV-1 and HSV-2 cloned DNA fragments. Specific hybridization to HSV-2 BglII N fragment was observed in six (9.4%) genital tumors. Labial and cerebral tumors did not show hybridization to any of the probes used. The technique employed allowed the detection of 0.1 copies of viral fragments per diploid genome.*

## INTRODUCTION

HERPES Simplex Virus type 1 and 2 (HSV-1 and HSV-2) are able to transform rodent cells to malignant phenotype [1] and have been proposed as etiologic agents in some human tumors [2].

HSV-2 has been incriminated as a possible cause of cervical cancer on the basis of serologic and epidemiologic studies [3]. Supporting evidence comes from finding HSV antigens expressed in tumor cells [4], as well as from the detection of viral RNA [5, 6] and DNA [7–10] in some human tumors. Moreover Wentz *et al.* [11] showed that repeated exposure of the mouse cervix to inactivated HSV-2 causes cervical dysplasia and invasive cancer. Even if the data available suggest an association between HSV-2 and genital cancer, further investigations will be needed before establishing a role of HSV-2 in oncogenesis.

Even if fewer studies have been undertaken on the relationship between HSV-1 and labial and cerebral tumors, yet some observations support their connection.

The rationale suggesting to search for a possible relationship between labial tumors and HSV-1 is mainly supported by two observations: (i) lips are the preferential site of a high percentage of herpetic recurrences and sometimes the tumor arises on the same site of herpetic recurrences [12]; (ii) even if sometimes patients with oral cancer show high antibody titers to HSV-1 [13], a significant epidemiologic association was not always detected between *Herpes labialis* and lip cancer risk [14, 15].

About the relationship between cerebral tumors and HSV-1, viral nucleic acids were detected in human brains by *in situ* hybridization, although no virus could be isolated, nor any viral antigen detected by immunofluorescence [16]. Furthermore, by Southern blot and dot-blot hybridization techniques, HSV-1 DNA sequences were found in brains of patients who died from neurological and non-neurological causes [17], and in post-mortem and operative specimens of temporal lobes from epileptic patients [18]. Viral DNA sequences were also found by reassociation kinetic analysis in brains of mice latently infected with HSV-1 [19].

Based on these reports, we took an interest in analysing a large number of human tumors arising in sites usually infected by HSV (genital, cerebral and labial tissues), by a technique able to detect 0.1 copies of viral DNA fragments per diploid genome. In this work 64 genital neoplasias were searched by Southern blot hybridization [20] for the presence of sequences homologous to HSV-2 DNA fragments, mainly BglII N, while 34 cerebral and 35 labial tumors were analysed for the presence of sequences homologous to HSV-1 DNA fragments, mainly BglII I. These fragments were chosen since both are able to transform cell cul-

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tures *in vitro* and do not have sequences homologous to cellular DNA [1]. Furthermore DNA sequences homologous to BglII N had already been detected in DNA from some genital tumors [8–10].

## MATERIALS AND METHODS

*Tumor samples*

Biopsy specimens or surgically removed material were obtained from patients enrolled at the Gynecologic, Dermatologic or Neurosurgical Clinics of the University of Ferrara. The samples were immediately frozen in liquid nitrogen, brought to the Institute of Microbiology and stored at −80° C until DNA extraction. Material immediately adjacent to each sample selected for us was subjected to hystopathologic examination. The samples were sent under code and the analysis was done without knowledge of the patients' diagnoses.

*Processing of DNA*

DNA was extracted from tissues and purified as already described by Cassai *et al.* [21]. Ten micrograms of DNA from each genital tissue were digested with EcoRI and SacI enzymes (6 units/μg of DNA for 4 hr at 37° C), while DNAs from labial or cerebral tissues were digested with XbaI and KpnI when probed to HSV DNA BglII fragments, with BamHI when assayed with HSV DNA BamHI fragments. Electrophoresis was performed on 0.6% agarose gels (1.5 V/cm for 15 hr). Transfer of DNA from the gels to hybridization membranes (Gene Screen Plus, NEN Research Products) was accomplished in 10X SSC (SSC 1X is 0.15 M Sodium Chloride and 0.015 M Sodium Citrate) for at least 14 hr. After transfer, the hybridization membranes were treated with 0.4 N NaOH to ensure complete denaturation of immobilized DNA, rinsed briefly in 0.2 M Tris–HCl pH 7.5-2X SSC, dried at room temperature and stored at 4° C.

*Plasmids*

HSV-2 (333) BglII fragments, cloned in pkC7, were a generous gift of N. Frenkel (University of Chicago, U.S.A.). HSV-1 (F) BamHI E, M and J, cloned in pBR322, were a generous gift of B. Roizman (University of Chicago, U.S.A.). HSV-1 BglII I was cloned in pkC7 in our laboratory.

*$^{32}P$ Labelling of probes*

The HSV DNA fragments cloned in the plasmid vector were labelled *in vitro* with $^{32}P$ dGTP and $^{32}P$ dCTP by nick translation reaction with a specific activity of about $1-3\times10^8$ cpm/μg of DNA. HSV-2 DNA BglII C (0.42–0.58 m.u.), BglII N (0.58–0.63 m.u.) and BglII I (0.63–0.72 m.u.) fragments were hybridized to DNAs from genital samples in high stringency conditions. To search HSV-1 DNA sequences in labial and cerebral tumors the probe used in all the cases was HSV-1 BglII I (0.31–0.41 m.u.). Some labial and cerebral tumors were analysed also for homology to HSV-2 BglII N, HSV-1 BamHI E (0.02–0.08 m.u.), M (0.22–0.25 m.u.) and J (0.88–0.92 m.u.).

*Hybridization*

The hybridization membranes were first incubated in prehybridization solution (30% deionized Formamide, 1% SDS, 1 M NaCl, 10% Dextran Sulfate) for 6 hr at 65° C; heat denatured $^{32}P$ labelled HSV DNA probes and salmon sperm DNA were added at the end of prehybridization period. Incubation was carried out for 18 hr at 65° C with constant agitation. Filters were rinsed twice with SSC 2X and SDS 0.1% for 30 min at 65° C, and extensively washed in decreasing concentrations of SSC (2X to 0.1X) at 65° C. Membranes were dried and autoradiographed, usually for 15 days, at −80° C with intensifying screens. To assess the sensitivity of the technique employed, the equivalent of 10, 1 and 0.1 copies of the cloned viral fragments used as probes were mixed with 10 μg of digested salmon sperm DNA, electrophoresed, transferred to hybridization membranes and hybridized to the corresponding $^{32}P$ labelled HSV DNA fragments. It was shown that the technique allowed the detection of 0.1 copies of viral sequences per diploid genome.

## RESULTS

*Analysis of DNA from genital tumors*

DNAs extracted from different tissues of the female lower genital tract were hybridized to $^{32}P$ labelled plasmid DNA containing HSV-2 DNA BglII N fragment; Table 1 shows the hystologic classification of the analysed tissues. Out of the 64 neoplasias analysed, three dysplasias (one vulvar and two cervical) and three invasive carcinomas (two vulvar and one cervical) contained sequences homologous to BglII N, while DNAs from 25 normal tissues were all negative. Figure 1 presents all the six positive samples obtained when the 64 genital tumors were probed with BglII N fragment (7.6 kb). The intensity of the radioactive bands compared with that obtained in reconstruction experiments suggested that the sequences homologous to BglII N were present in 0.1–0.5 copies (lanes B, D, E, F) and in 5–10 copies (lanes A and C) per diploid genome.

EcoRI/SacI digestion of BglII N, generates four hybridizable fragments of respectively 0.6, 1.5, 2.2, 3.1 kb. The DNAs of samples A–E showed a single hybridization band with the apparent size

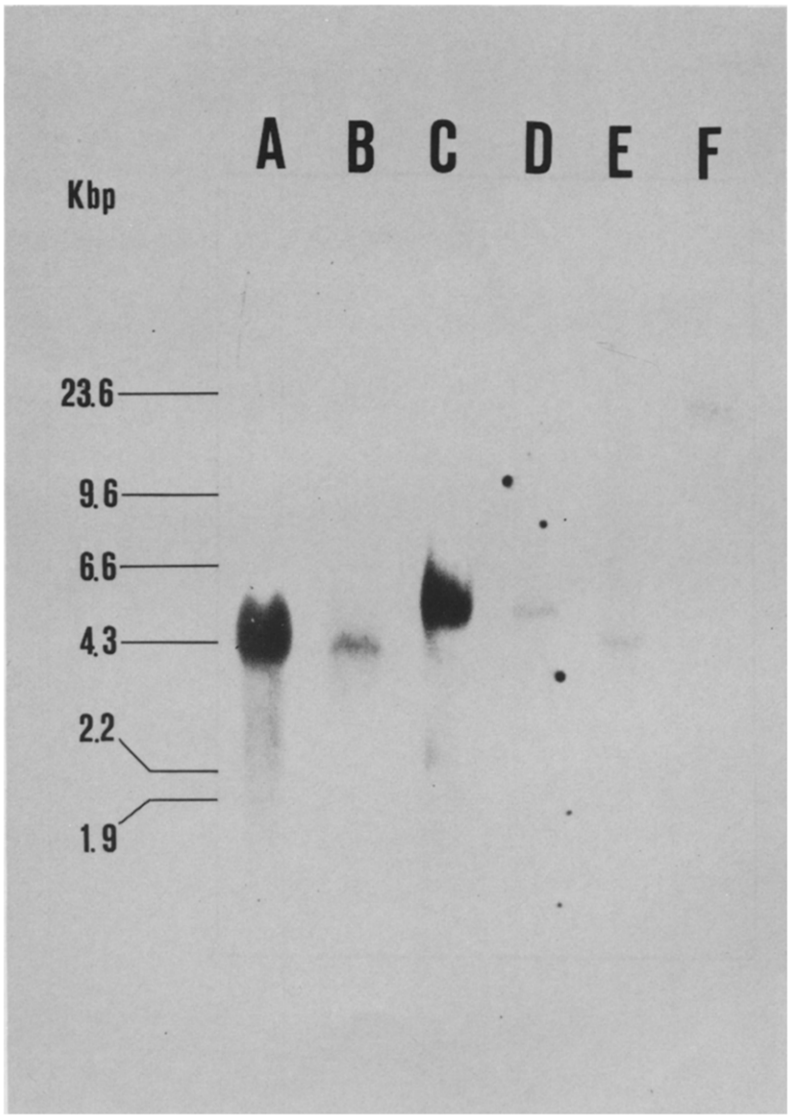


*Fig. 1. Autoradiogram of Southern blot hybridization reaction of DNA extracted from the six genital tumors positive to the BGlII N probe. The size markers are Hind III fragments of phage lambda DNA. Lanes A and D contained DNA from cervix dysplasia, lane E contained DNA from vulvar dysplasia, while lane C contained DNA from cervix invasive carcinoma and lanes B and F contained DNA from vulva invasive carcinoma.*

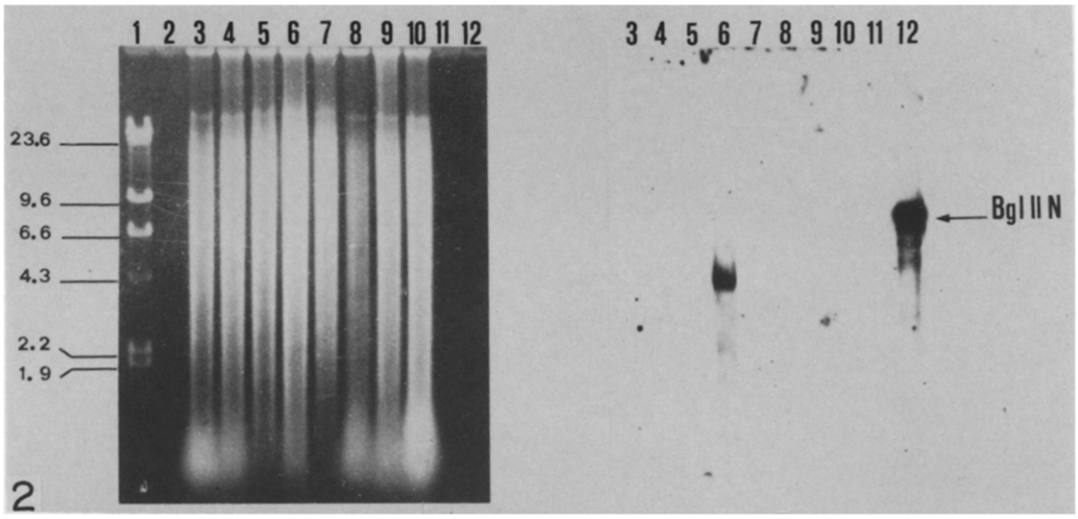


*Fig. 2. Detection of sequences homologous to HSV-2 DNA fragment BglII N. On the left is shown the ethidium bromide stained pattern of the gel, on the right the autoradiogram of the hybridized filter. DNA* (10 μg/lane) *from two control cervical tissues (lanes* 3, 10), *four invasive cervical carcinomas (lanes* 4–7), *two cervical dysplasias (lanes* 8, 9), *were cleaved by a double digestion EcoRI/SacI, electrophoresed through* 0.6% *agarose, transferred to nitrocellulose and hybridized to* $^{32}$P *labelled plasmid containing HSV-2 BglII N fragment and exposed for autoradiography. Lane* 1 *contains HindIII cleaved phage lambda DNA, lanes* 2 *and* 11 *do not contain any sample, lane* 12 *contains the equivalent of* 10 *copies of HSV-2* (33) *DNA cut with BglII. The positive sample in lane* 6 *corresponds to sample C in Fig.* 1.

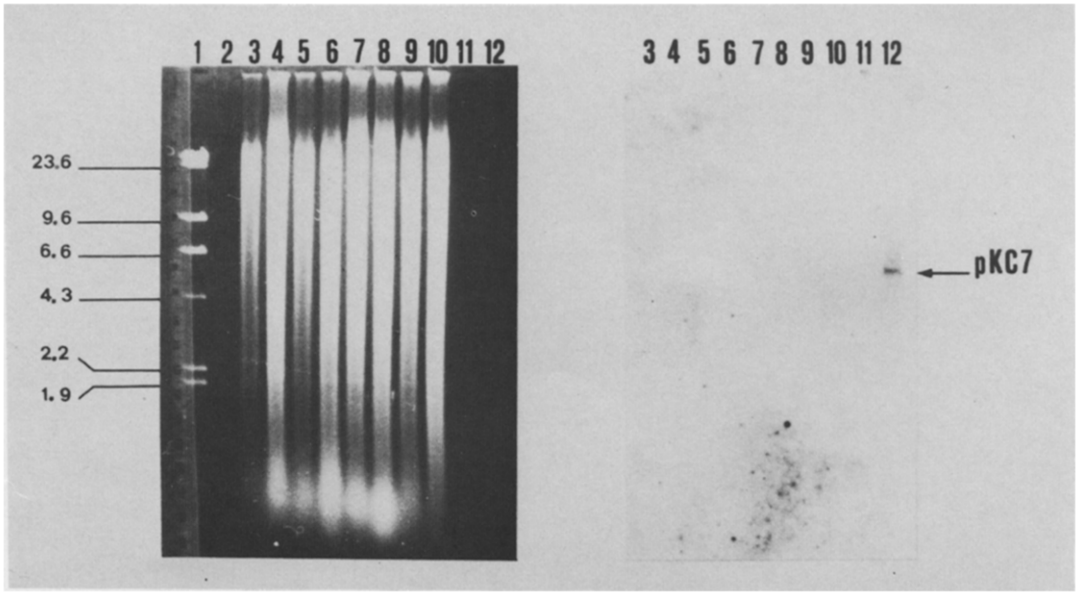


*Fig. 3. Hybridization test using pKC7 DNA as probe. On the left is shown the ethidium bromide stained pattern of the gel, on the right the autoradiogram of the hybridization filter. All samples, the same of Fig.* 2, *were digested with BglII, electrophoresed through* 0.6% *agarose, transferred to nitrocellulose and hybridized to* $^{32}$P *labelled plasmid pKC7 and exposed for autoradiography. Lane* 1 *contains HindIII cleaved phage lambda DNA, lanes* 2 *and* 11 *do not contain any samples, lane* 12 *contains the equivalent of* 0.2 *copies of pKC7 DNA cut with BglII.*

*Table* 1. *HSV-2 BglII N fragment in neoplasias of the lower genital tract*

| | | Total no. specimens | No. positive samples |
|---|---|---|---|
| Dysplasia | Vulva | 4 | 1 |
| | Cervix | 22 | 2 |
| *In situ* carcinoma | | 5 | 0 |
| Invasive carcinoma | Vulva | 14 | 2 |
| | Cervix | 19 | 1 |
| Total neoplastic specimens | | 64 | 6 (9.4%) |
| Controls (cervical/vulvar non neoplastic lesions; normal cervical/vulvar tissue from cancer patients). | | 25 | 0 |

of 4.3–5 kb, whereas sample F showed a band of about 20 kb, indicating that only a part of BglII N is present, presumably integrated. Figure 2 shows one of the hybridization experiments where one positive sample was detected out of eight analysed for the presence of BglII N sequences.

Since the HSV DNA fragments used as probes were not purified from plasmid DNA before labelling, all the samples were also separately hybridized to a pkC7 probe, in order to exclude the possibility of false positive results due to cross-hybridization of labelled plasmid DNA to host DNA or to DNA from bacteria of the genital flora. None of the tumor or control DNA showed homology to plasmid DNA; Fig. 3 shows one of these hybridization experiments.

DNAs from six cervical invasive carcinomas, three vulvar dysplasias and one cervical dysplasia (this last sample being positive to BglII N), were probed in high stringency conditions also to HSV-2 BglII C DNA fragment which is reported to have transforming activity for cultured cells [22]. Moreover, DNAs of two vulvar invasive carcinomas and one vulvar dysplasia positive to BglII N, were probed to the HSV-2 BglII I fragment. In no case hybridization bands were detected (data not shown).

Since HSV-1 has been consistently isolated from herpetic genital infections, the DNAs from 11 neoplasias (six invasive carcinomas from cervix and five from vulva), all negative to HSV-2 BglII N fragment, were probed to HSV-1 BglII I transforming fragment, but no hybridization bands were detected.

*Analysis of DNA from labial and cerebral tumors*

DNAs extracted from 34 cerebral tumors and 35 labial tumors (Table 2) were probed to HSV-1 DNA BglII I fragment by Southern blot hybridization. DNAs from eight cerebral tumors and eight labial tumors were hybridized also to HSV-1 DNA fragments BamHI J, E, M and to HSV-2 DNA fragment BglII N which cross-hybridizes with the corresponding region of HSV-1 DNA. Other 16 labial tumors were tested with a HSV-2 BglII N probe. All these experiments yielded negative results.

*Table* 2. *Tissues analysed for the presence of HSV-1 DNA sequences*

| Cerebral tissues | | Labial tissues | |
|---|---|---|---|
| Diagnosis | No. | Diagnosis | No. |
| Meningioma | 16 | Spinocellular epithelioma | 28 |
| Astrocytoma | 6 | Keratoacanthoma | 7 |
| Glioblastoma | 6 | | |
| Oligodendroglioma | 3 | | |
| Spongioblastoma | 1 | | |
| Neurinoma | 1 | | |
| Meningosarcoma | 1 | | |
| Total | 34 | Total | 35 |

## DISCUSSION

Frenkel *et al.* [7] detected HSV-2 DNA in an invasive cervical carcinoma. Attempts to repeat this result have been unsuccessful [23–25], probably because the complexity of the whole virus DNA probe is so great that persistence of a small portion of the genome would not be detected in such experiments. Recently more uniform results were obtained when the DNAs extracted from cervical dysplasias and carcinomas were subjected to Southern blot analysis and hybridized to specific fragments of viral DNA. Several authors detected sequences homologous to fragments of the HSV-2 genome: in 3 out of 9 [8], in 1 out of 8 [9] and in 2 out of 13 cervical cancers [10]. However these studies were affected by the limitation of the small number of tumors analysed and in some cases by the lack of adequate controls.

In the high stringency conditions employed in this study (65° C in 30% Formamide), 6 out of 64 vulvar and cervical neoplasias had sequences homologous to BglII N fragment, whereas none of the 25 normal tissues, which were often obtained from areas surrounding the neoplastic tissue, was positive.

The fact that sequences homologous to only a part of BglII N fragment are present in these tumors could be in agreement with the data obtained by Galloway *et al.* [26] who succeeded in transforming rodent cell cultures *in vitro* with a portion of BglII N of 737 base pairs. More information on the state of the sequences homologous to BglII N will be obtained only by cloning the DNA sequences homologous to HSV with the flanking cellular sequences.

Few studies focused on the possible association between HSV-1 and human tumors. In the past [21] we looked for HSV-1 DNA in human labial tumors by Southern hybridization technique. None of the tumors tested showed sequences homologous to HSV-1 DNA sequences, but the analysis employed the whole viral genome as probe and the technique could not evidence less than 0.5 copies of the viral genome per diploid cell genome. In the present study we analysed both labial and cerebral tumors with a detection level of 0.1 copies of HSV DNA fragments per diploid cell genome, obtaining negative results. However these data are not yet definitive; more precise considerations will be possible only when DNAs from these and other tumors are tested with all the HSV DNA fragments (work in progress). Since HSV harbors in neurons in a latent state [27], special attention needs to be given to tumors specific of neuronal cells.

The data presented in this paper suggest that the relationship between genital tumors and HSV is not casual. This observation is supported by the presence of HSV DNA sequences in vulvar and in cervical neoplasias, but not in tumors arisen in anatomical sites often infected by HSV (such as brain and lip). The low incidence of genital tumors containing sequences homologous to BglII N (9.4%) can be explained by the following considerations: (i) the high stringency conditions of hybridization employed. (ii) HSV-2 fragments other than BglII N could be present. (iii) Since in the DNAs of the two HSV types are present regions of detectably lower homology [28], also HSV-1 should be considered as a factor in the genital tumor oncogenesis; in fact in our region about 40% of HSV isolated from the female genital tract are characterized as HSV-1 (E. Cassai, unpublished observations). (iv) The "hit and run" hypothesis [8] could account for the absence of viral sequences in most tumors. Alternatively, HSVs could interact synergistically with other initiating or promoting factors involved in multistep carcinogenesis [29]. About this last point, attention should be given to Human Papillomaviruses, especially types 11, 16 and 18, which DNAs are found associated to human genital neoplasias [30]. It was shown that Papillomavirus infection is not sufficient to induce tumor formation and that tumor growth requires the synergistic action of cocarcinogenetic factors [31]; the synergism between HSV and Papillomaviruses could be a condition for genital tumor development. For this purpose both HSV and Human Papillomavirus footprints should be searched in the same pathological sample.

## REFERENCES

1. Hayward GS, Reyes GR. Biochemical aspects of transformation by Herpes Simplex Viruses. *Adv Viral Oncol* 1983, **3,** 271–306.
2. Rawls WE. Herpes Simplex Viruses and their role in human cancer. In: Roizman B, ed. *The Herpesviruses.* New York and London, Plenum Press, 1985, Vol. 3, 241–255.
3. Rawls WE, Campione-Piccardo J. Epidemiology of Herpes Simplex Virus type 1 and type 2 infections. In: Nahmias AJ, Dowdle WR, Schinazi RE, eds. *The Human Herpes Viruses: An Interdisciplinary Perspective.* New York, Elsevier, 1981, 137–152.
4. Aurelian L. Herpes Simplex Virus type 2 and cervical cancer. *Clinics in Dermatol* 1984, **2,** 90–99.
5. McDougall JK, Fenoglio CM, Galloway DA. Cervical carcinoma: Detection of Herpes Simplex Virus RNA in cells undergoing neoplastic change. *Int J Cancer* 1980, **25,** 1–8.

6. Eglin RP, Sharp F, MacLean AB, Macnab JCM, Clements JB, Wilkie NM. Detection of RNA complementary to Herpes Simplex Virus DNA in human cervical squamous cell neoplasms. *Cancer Res* 1981, **41,** 3597–3603.
7. Frenkel N, Roizman B, Cassai E, Nahmias A. A DNA fragment of Herpes Simplex 2 and its transcription in human cervical cancer tissue. *Proc Natl Acad Sci USA* 1972, **69,** 3784–3789.
8. Galloway DA, McDougall JK. The oncogenic potential of Herpes Simplex Viruses: evidence for a "hit and run" mechanism. *Nature* 1983, **302,** 21–24.
9. Park M, Kitchener HC, Macnab JCM. Detection of Herpes Simplex Virus type 2 DNA restriction fragments in human cervical carcinoma tissue. *EMBO J* 1983, **2,** 1029–1034.
10. Prakash SS, Reeves WC, Sisson GR *et al.* Herpes Simplex Virus Type 2 and human papillomavirus Type 16 in cervicitis, dysplasia and invasive cervical carcinoma. *Int J Cancer* 1985, **35,** 51–57.
11. Wentz WB, Reagan JW, Heggie AD, Fu YS, Anthony DD. Induction of uterine cancer with inactivated Herpes Simplex Virus types 1 and 2. *Cancer* 1981, **48,** 1783–1790.
12. Kvasnicka A. Relationship between Herpes Simplex and lip carcinoma. III. *Neoplasma* 1963, **10,** 199–203.
13. Shillitoe EJ, Greenspan D, Greenspan JS, Silverman S Jr. Antibody to early and late antigens of Herpes Simplex virus type 1 in patients with oral cancer. *Cancer* 1984, **54,** 266–273.
14. Lindqvist C. Risk factor of lip cancer: a critical evaluation based on epidemiological comparisons. *Am J Publ Health* 1979, **69,** 256–260.
15. Dardanoni L, Gafà L, Paternò R, Pavone G. A case-control study on lip cancer risk factors in Ragusa (Sicily). *Int J Cancer* 1984, **34,** 335–337.
16. Sequiera LW, Jennings LC, Carrasco LH, Lord MA, Curry A, Sutton RNP. Detection of Herpes Simplex viral genome in brain tissue. *Lancet* 1979, **II,** 609–612.
17. Fraser NW, Lawrence WC, Wroblewska Z, Gilden DH, Koprowski H. Herpes Simplex type 1 DNA in human brain tissue. *Proc Natl Acad Sci USA* 1981, **78,** 6461–6465.
18. Gannicliffe A, Saldanha JA, Itzhaki RF, Sutton RNP. Herpes Simplex Viral DNA in temporal lobe epilepsy. *Lancet* 1985, **I,** 214–215.
19. Cabrera CV, Wohlenberg C, Openshaw H, Rey-Mendez M, Puga A, Notkins AL. Herpes Simplex virus DNA sequences in the CNS of latently infected mice. *Nature* 1980, **288,** 288–290.
20. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975, **98,** 503–517.
21. Cassai E, Rotola A, Di Luca D *et al.* Herpes Simplex Virus and human cancer. II. Search for relationship between labial tumours and Herpes Simplex type 1. *Eur J Cancer* 1981, **17,** 695–702.
22. Jariwalla RJ, Aurelian L, Ts'O POP. Immortalization and neoplastic transformation of normal diploid cells by defined cloned DNA fragments of Herpes Simplex Virus type 2. *Proc Natl Acad Sci USA* 1983, **80,** 5902–5906.
23. zur Hausen H, Schulte-Holthausen H, Wolf H, Dorries K, Egger H. Attempts to detect virus-specific DNA in human tumors: II. Nucleic acid hybridization with complementary RNA of human Herpes group viruses. *Int J Cancer* 1974, **13,** 657–664.
24. Pagano JS. Disease and mechanisms of persistent DNA virus infection: latency and cellular transformation. *J Dis* 1975, **132,** 209–223.
25. Cassai E, Rotola A, Meneguzzi G *et al.* Herpes Simplex Virus in human cancer. I. Relationship between human cervical tumors and Herpes Simplex type 2. *Eur J Cancer* 1981, **17,** 685–693.
26. Galloway DA, Nelson JA, McDougall JK. Small fragments of Herpesvirus DNA with transforming activity contain insertion sequence-like structures. *Proc Natl Acad Sci USA* 1984, **81,** 4736–4740.
27. Cook ML, Bastone VB, Stevens JG. Evidence that neurons harbor latent Herpes Simplex Virus. *Infect Immun* 1974, **9,** 946–951.
28. Draper KG, Frink RJ, Devi GB, Swain M, Galloway D, Wagner EK. Herpes Simplex Virus type 1 and 2 homology in the region between 0.58 and 0.68 map units. *J Virol* 1984, **52,** 615–623.
29. zur Hausen H, Gissmann L, Schlehofer JR. Viruses in the etiology of human genital cancer. *Prog Med Virol* 1984, **30,** 170–186.
30. Crawford L. Papillomaviruses and cervical tumours. *Nature* 1984, **310,** 16.
31. Gissman L. Papillomaviruses and their association with cancer in animals and in man. *Cancer Surv* 1984, **3,** 161–181.