0022-202X/84/8301S-0026s$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 83:26s–28s, 1984

Vol. 83, No. 1 Supplement
Printed in U.S.A.

Presence of Human Papillomavirus in Genital Tumors

LUTZ GISSMANN, MICHAEL BOSHART, MATTHIAS DÜRST, HANS IKENBERG, DIETER WAGNER, AND HARALD ZUR HAUSEN

Deutsches Krebsforschungszentrum, Intitute für Virusforschung, Heidelberg (LG, HH), Institut für Virologie, Freiburg/Breisgau (MB, MD, HI), Institut für Klinische Virologie, Erlangen (MB), and Cytologisches Labor au Evang. Diakoniekrankenhang, Freiburg/Breisgau (DW), Federal Republic of Germany.

Human papillomavirus (HPV) types 16 and 18 have been identified in two different human cervical carcinomas. The viral DNAs were molecularly cloned and used as probes to screen a large number of genital tumors by Southern blot analysis. HPV-16 or HPV-18 sequences, respectively, were found in a high percentage of cervical carcinomas, but only in a small number of condylomata acuminata or flat condylomas. The majority of the latter lesions, however, contained HPV-6 or HPV-11 sequences, respectively, which in contrast were detected only rarely in carcinomas in situ or invasively growing carcinomas. A similar distribution of the different papillomaviruses was observed when cell swabs taken from the cervix were tested by in situ hybridization.

The involvement of an infectious event in the development of human cancer of the uterine cervix has been implicated for many years. This was mainly based on epidemiologic studies that showed that the time of first intercourse and the number of sexual partners are most important factors associated with cervical neoplasia [1]. Women whose husbands have multiple sexual partners are also at relatively higher risk [2]. Besides other agents, herpes simplex virus (HSV) has been extensively investigated for its association with cervical carcinomas. Serologic data in fact support the hypothesis that this virus might play a role in tumor development, but almost all attempts to detect viral DNA within the biopsies gave negative results [3]. However, human papillomaviruses have been discussed as possible candidates for a few years [4] for the following reasons:

1. The oncogenic potential of some papillomaviruses, e.g., of the Shope papillomaviruses and the bovine papillomavirus, is well documented [5,6].
2. An eventual malignant conversion of certain virus-induced human papillomas (e.g., laryngeal papillomas, genital warts, lesions in epidermodysplasia verruciformis patients) has been reported [4,6].
3. Venerally transmitted papillomavirus infections of the genital tract are very frequent. Approximately 2% of unselected women were shown to be affected by genital papillomas [7].
4. Mainly due to investigations by Meisels and coworkers it became clear that cervical lesions that have been diagnosed as mild dysplasia are in fact papillomavirus-associated condylomas [8,9]. It is known, however, that these dysplasias progress with a certain probability to cervical intraepithelial neoplasia grades II and III and to invasive cancer.

Human papillomaviruses represent a very heterogeneous group. At least 24 different virus types have been identified thus far [10] that are found in particular lesions and whose genomes show only a limited, if any, sequence homology with each other. Considering this property, it is not surprising that the first attempts to detect papillomavirus DNA in genital tumors have been unsuccessful, since DNA prepared from skin wart viruses has been used as probe in these hybridization experiments [11,12].

The concentration of papillomavirus particles in genital warts is extremely low. Since no permissive cell system for virus replication has been established thus far [5], molecular cloning of the DNA was a prerequisite in order to investigate its presence in different tumors. By centrifugation in a cesium chlorid–ethidium bromide equilibrium gradient, the supercoiled viral DNA was purified directly from the total cellular DNA derived from a genital wart without prior purification of virus particles [13]. The yield of viral DNA obtained by this method was in some tumors 10 to 50 ng, which was sufficient for molecular cloning in the *E. coli* plasmid pBR322 [14].

The cloned DNA was tested by hybridization with DNA extracted from virus particles that had been purified from the same genital wart. Because of the high sensitivity of DNA hybridization (only 10^6 molecules are necessary), the low concentration of papillomavirus present in genital warts was sufficient to clearly identify the cloned DNA as the papillomavirus genome [10].

Comparison with the other HPV types by restriction enzyme analysis and by hybridization indicated this DNA to be a new papillomavirus, designated as HPV-6 [13]. The DNA of a closely related virus, HPV-11, which was first observed in a laryngeal papilloma, was cloned from a genomic library in bacteriophage λ [15]. The HPV DNA-positive plaques were identified by in situ hybridization using HPV-6 DNA as a probe. HPV-6 and HPV-11 DNA were cleaved from the vector by the respective restriction endonuclease, purified in agarose gels, and radioactively labeled with ^{32}P-TTP by nick translation. For Southern blot experiments, cellular DNA extracted from different clinical biopsies was cleaved with restriction enzymes, run through an agarose gel, denatured by alkaline treatment, transferred onto a nitrocellulose filter, and incubated with the labeled HPV DNA with different concentrations of formamide (see below), 0.8 *M* Na^+ at 42°C.

Using stringent conditions of hybridization (50% formamide corresponding to 20°C below T_m), it has been shown that the majority of genital warts (96 of 106 cases tested) contained sequences of HPV-6 in two-thirds and HPV-11 in one-third of the cases ([16,17] and Ikenberg, Gissmann, and zur Hausen, unpublished data). Both viruses are closely related [15], and no type-specific differences in clinical appearance or histologic picture could be observed between the individual papillomas containing one or the other virus (Gross, Ikenberg, and Gissmann, in preparation). Therefore, in the following, the respective lesions will be classified as HPV-6 or HPV-11 positive. Forty-two percent of flat condylomas associated with dysplasias of different severity contained HPV-6 or HPV-11, which have been found only once in 29 cases of cervical carcinoma biopsies tested thus far ([17] and Ikenberg, Gissmann, and zur Hausen, unpublished data).

As stated earlier, many of the different human papillomavirus

Reprint requests to: Dr. Lutz Gissmann, Deutsches Krebsforschungszentrum, Institute für Virusforschung, Im Neuenheimer Feld 280, D-6900 Heidelberg 1, Federal Republic of Germany.

Abbreviations:
HPV: human papillomavirus
HSV: herpes simplex virus

DNAs do not cross-react with each other under hybridization conditions of high stringency (20°C below the T_m). If the temperature of hybridization is lowered to 40°C below the T_m (20% formamide, 0.8 M Na^+, 42°C), one can detect papillomavirus sequences of even more distantly related types ([18] and Gissmann, unpublished data). Using these conditions, two thus far unknown human papillomaviruses were identified by Southern blot hybridization in two different cervical carcinomas [19,20]. After cloning, these DNAs were analyzed by restriction enzyme analysis and found to be different from the other papillomaviruses described thus far.

Although these molecules have never been detected in papillomavirus particles, it seems to be justified to designate them as viral DNA [10], since

1. They have the same size (approximately 8 kb) as papillomavirus DNA.
2. At least in some tumors they persist as 8-kb circular DNA, i.e., as a molecule which theoretically can be packaged into virus particles.
3. They hybridize with other papillomaviruses under nonstringent conditions.
4. They are colinear with well-characterized human papillomaviruses, as shown by hybridization of single fragments.

The new isolates from cervical carcinomas have been labeled as HPV-16 and HPV-18, respectively. They were used as probes to screen additional tumors by Southern blot analyses under stringent conditions of hybridization. It turned out that HPV-16 or HPV-18, respectively, were present in 6.1% of condylomata acuminata, 16.7% of flat condylomas, but in 53.8% of carcinomata in situ and in 57.4% of cervical cancers ([19,20] and Ikenberg, Gissmann, and zur Hausen, unpublished data). Using the in situ hybridization technique on frozen sections of four different carcinomas, it was shown that the specific label is restricted to the tumor cells (Grussendorf-Conen, Ikenberg, and Gissmann, submitted). The data clearly show a preferential association of HPV-16 and HPV-18 with malignant tumors and might indicate an elevated risk for patients infected with these two viruses.

It should be mentioned here that HPV-16 could be detected in 7 of 10 cervical dysplasias containing so-called abnormal mitotic figures as indications for a premalignant lesion [21]. When the negative cervical carcinomas were tested under nonstringent conditions, 69.6% were shown to contain papillomavirus sequences, indicating the presence of at least one additional thus far uncharacterized virus type in these tumors. Thus approximately 90% of cervical carcinoma biopsies harbor HPV sequences.

A similar distribution of different papillomaviruses was found when epithelial cells of the cervix regularly taken for routine Papanicolaou smears were hybridized in situ with HPV-11 and with a mixture of HPV-16 and HPV-18 (Gissmann, Wagner, Ikenberg, and Böhm, submitted). Approximately 10^4 to 10^6 epithelial cells were spotted onto a nitrocellulose filter. They were treated with alkaline to fix the cells and to denature the DNA, which was then hybridized with the ^{32}P-labeled HPV probes under stringent conditions.

No positive reaction with HPV-16 and HPV-18 was seen in 63 different patients with normal Pap smears, but in 6 of 13 patients cytologically diagnosed as having mild or moderate dysplasia and in 15 of 22 carcinomas in situ, HPV-6 or HPV-11 sequences were found at frequencies of 4 in 36, 6 in 13, and 4 in 22, respectively. It will be of interest to follow up those patients in whom a clear diagnosis from the Pap smear could not be set up but who were positive for HPV-11 or HPV-16 and HPV-18, respectively. One could expect that only in the HPV-16 and HPV-18 positive cases would definite dysplastic changes be observed within the next few months.

The rare occurrence of HPV-16 and HPV-18 DNA in condylomata acuminata or flat condylomas, respectively, excludes the possibility that the DNA derived from carcinomas that contain these sequences might be contaminated by adjacent genital warts. However, it does raise the question as to which primary lesions are induced by HPV-16 or HPV-18, especially in the male population. It is attractive to speculate that the very often inconspicous but quite frequently occuring Bowenoid papules at the glans penis or the vulva (G. Gross, personal communication) might represent the reservoir for virus spread, at least in case of HPV-16, since viral sequences of this type could be found in the majority of such Bowenoid papulosis lesions [22].

The state of the viral DNA within the tumor cells has been investigated by cesium chloride–ethidium bromide centrifugation and two-dimensional electrophoresis. There is evidence that in cervical carcinomas the HPV-16 or HPV-18 DNA, respectively, is covalently linked to the cellular genome [20]. This contrasts with the situation in genital warts, where the HPV-16 molecules persist exclusively in a nonintegrated form. It is an interesting question whether the physical state of the viral DNA within a given cell is of influence for malignant growth. The high frequency of particular papillomaviruses within human genital carcinomas does, of course, per se not prove their causative role in tumor development. Epidemiologic data clearly indicate the involvement of other factors, e.g., herpes simplex virus infection, which might act synergistically with those viruses in cell transformation [23]. The present knowledge of specific virus-cell interactions, however, is still refractory, and additional studies on the biological features of the papillomaviruses in vivo and in vitro are required to improve our understanding of their role in human genital cancer.

REFERENCES

1. Thomas DB: An epidemiologic study of carcinoma in situ and squamous dysplasia of the uterine cervix. Am J Epidemiol 98:10–28, 1973
2. Martin CE: Epidemiology of cancer of the cervix. II. Marital and coital factors in cervical cancer. Am J Public Health 57:803–814, 1967
3. zur Hausen H: Herpes simplex virus in human genital cancer. Int Rev Exp Pathol, 25:307–325, 1983
4. zur Hausen H: Human papillomaviruses and their possible role in squamous cell carcinomas. Curr Top Microbiol Immunol 78:1–30, 1977
5. Pfister H: Biology and biochemistry of papillomaviruses. Rev Physiol Biochem Pharmacol 99:111–181, 1984
6. Gissmann L: Papillomaviruses and their assoziation with cancer in animals and in man. Cancer Surveys, in press
7. Meisels A, Morin C, Casas-Codero M: Human papillomavirus infection of the uterine cervix. Int J Gynecol Pathol 1:75–94, 1982
8. Meisels A, Fortin R: Condylomatous lesions of the cervix and vagina. I. Cytological patterns. Acta Cytol 20:505–509, 1976
9. Purola E, Savia E: Cytology of gynecologic condyloma acuminatum. Acta Cytol 21:26–31, 1977
10. Gissmann L, Schwarz E: Cloning of papillomavirus DNA, Developments in Molecular Virology, vol 5: Recombinant DNA. Edited by Y Becker. Hingham, Mass, Martinus Nijhoff, in press
11. zur Hausen H, Meinhof W, Bornkamm GW: Attempts to detect virus-specific DNA sequences in human tumors. I. Nucleic acid hybridization with complementary RNA of human wart virus. Int J Cancer 13:650–656, 1974
12. Gissmann L, Pfister H, zur Hausen H: Human papillomaviruses (HPV): Characterization of four different isolates. Virology 76:569–580, 1977
13. Gissmann L, zur Hausen H: Partial characterization of viral DNA from human genital warts (condylomata acuminata). Int J Cancer 29:143–146, 1980
14. de Villiers EM, Gissmann L, zur Hausen H: Molecular cloning of viral DNA from human genital warts. J Virol 40:932–935, 1981
15. Gissmann L, Diehl V, Schultz-Coulon HJ, zur Hausen H: Molecular cloning and characterization of human papillomavirus DNA derived from a laryngeal papilloma. J Virol 44:393–400, 1982
16. Gissmann L, de Villiers EM, zur Hausen H: Analyses of human genital warts (condylomata acuminata) and other genital tumors for human papillomaviruses type 6 DNA. Int J Cancer 29:140–143, 1982
17. Gissmann L, Wolnik L, Ikenberg H, Koldovsky U, Schnürch HG, zur Hausen H: Human papillomavirus type 6 and 11 DNA

sequences in genital and laryngeal papillomas and in some cervical cancers. Proc Natl Acad Sci USA 80:560–563, 1983
18. Howley PM, Law MF, Heilman C, Engel L, Alfonso MC, Israel MA, Lowy DR: Molecular characterization of papillomavirus genomes, Viruses in Naturally Occurring Cancers, Vol. 7. Edited by M Essex, G Todaro, H zur Hausen. Cold Spring Harbor, N.Y., Cold Spring Harbor Conferences on Cell Proliferation, 1980.
19. Dürst M, Gissmann L, Ikenberg H, zur Hausen H: A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsies form different geographic regions. Proc Natl Acad Sci USA 60:3812–3825, 1983
20. Boshart M, Gissmann L, Ikenberg H, Uleinheinz A, Scheurlen W, zur Hausen H: A new type of papillomavirus DNA: Its presence in genital cancer biopses and in cell lines derived from cervical cancer. EMBO J, in press
21. Crum CP, Ikenberg H, Richart RM, Gissmann L: Human papillomavirus type 16 and early cervical neoplasia. N Engl J Med 310:880–883, 1984
22. Ikenberg H, Gissmann L, Gross G, Grussendorf-Conen EI, zur Hausen H: Human papillomavirus type 16 related DNA in genital Bowen's disease and in Bowenoid papulosis. 32:563–566, 1983
23. zur Hausen H: Human genital cancer—Synergism between two virus infections or synergism between a virus infection and initiating events. Lancet 2:1370–1372, 1982