HUMAN PAPILLOMAVIRUS INFECTION AND CERVICAL CANCER IN LATIN AMERICA

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Abstract To evaluate a possible association between infection with human papillomavirus (HPV) and cervical cancer, we performed a multicenter case–control study in Latin America of 759 cases of invasive cervical cancer and 1467 randomly selected age-matched controls. Demographic, sexual, behavioral, and other clinical data were obtained by interview, and HPV DNA was assayed in cervical-swab specimens with use of filter in situ hybridization.

Cervical infection with HPV 16 or 18 or both was strongly associated with cervical cancer. HPV DNA was detected in 62 percent of the cases but only 32 percent of the controls, and the relative risk of cancer increased from 2.1 (95 percent confidence interval, 1.6 to 2.8) to 9.1 (6.1 to 13.6) with hybridization reactions of increasing strength. Although the number of sexual partners, age at first intercourse, number of live births, and Pap-smear history were also significant risk factors, the strong associations between infection with HPV 16 or 18 or both and cervical cancer persisted after we adjusted for these variables.

These observations are consistent with the hypothesis that genital infection with HPV 16 or 18 may have a role in the pathogenesis of cervical cancer. Other well-known risk factors were also identified in the study, but they did not affect the association between HPV and cervical cancer. (N Engl J Med 1989; 320:1437-41.)

EVIDENCE derived from molecular biology and clinical investigations has established an association between specific types of human papillomavirus (HPV) and cancer of the cervix.[1-7] DNA from HPV 16, HPV 18, or both (hereafter, HPV 16/18) has been found in the majority of cases of cervical cancer examined, and specific viral mechanisms have been postulated to account for the malignant conversion of these tumors.[1-3] However, there is insufficient information in epidemiologic studies to support strongly the hypothesis that HPV causes invasive cervical cancer. For example, a study of the presence of HPV 16/18 DNA in women from populations with markedly different cancer rates failed to show differences in viral prevalence.[4] In addition, two small case–control studies that adjusted for age and other relevant risk factors have revealed only slight relative risks in association with the detection of DNA from HPV 16/18.[5,6]

A number of other risk factors for cervical cancer have been identified — sexual behavior, exposure to sexually transmitted agents, socioeconomic factors, Pap-smear screening history, smoking, and the use of oral contraceptives.[7] The relations of these risk factors to the presence of HPV are unknown. It is also unclear to what extent factors such as smoking or oral contraceptives may enhance the effects of HPV.[1] To assess these relations, we undertook a large multicenter study, involving incident cases of invasive cervical cancer and matched controls from four high-risk areas in Latin America.

METHODS

Study Populations

Cases

We enrolled patients with invasive cervical cancer at four study sites: the Instituto Nacional de Oncología in Panama, to which 90 percent of all such patients in that country are ultimately referred[8]; the tertiary care hospitals (San Juan de Dios, Calderon Guardia, and Hospital Mexico) of the Caja Costarricense de Seguro Social Unidad Nacional de Cancerología in San Jose, Costa Rica, to which 80 percent of all Costa Rican patients with invasive cervical cancer are referred[9]; the Instituto Nacional de Cancerología in Bogotá, Colombia, which is responsible for all medically indigent patients with cancer in that city; and the Hospital de Oncología Nacional, Instituto Mexicano de Seguro Social, in Mexico City, which is re-

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sponsible for all patients with cancer who have social security coverage and live in Mexico City.

All new patients presumed to have invasive cervical cancer who presented to a collaborating center from January 1986 through June 1987 were identified. When her diagnosis (on the basis of biopsy) and eligibility were confirmed, each woman was invited to participate in the study. Eligible subjects were between 18 and 69 years of age, had newly diagnosed cervical cancer with no previous treatment, and had resided in the catchment area for at least six months.

Controls

Two controls, matched according to five-year age group, were enrolled for each case patient. In Bogotá and Mexico, both controls were selected from among patients at hospital inpatient services or outpatient clinics. In Panama and Costa Rica, one control was selected from the hospital and a second was selected from the general population.

In Panama and Costa Rica, the hospital controls were selected from the hospital that had referred the patient. In Bogotá, they were selected from among eight tertiary-level government hospitals that refer patients to the Instituto Nacional de Cancerología. In Mexico City, the hospital controls were selected from among three social security hospitals serving the population from which the cases were derived. The hospital controls were selected randomly from lists of current patients. Once the control patient was selected, her chart was reviewed to determine eligibility. Women under evaluation for gynecologic or endocrine diseases or diseases related to smoking, who had a previous diagnosis of cancer or who had had a hysterectomy were ineligible and were replaced in the study population. Eligible controls who chose not to participate were not replaced.

Controls from the community in Panama and Costa Rica were selected randomly from census lists of women with the same county of residence and age group as the case patient. We visited the potential controls at their homes; if a woman had had a hysterectomy or a previous diagnosis of cervical cancer, another control was selected. The women who chose not to participate as controls were not replaced.

Although the women with a previous diagnosis of cervical cancer and those under evaluation for specified conditions at hospitals were ineligible as controls, the existence of previously unrecognized gynecologic disease was not a criterion for exclusion. Sixteen controls (1.2 percent) had cytologic evidence of cervical intraepithelial neoplasia on examination after enrollment. They were retained in the study.

Seven hundred sixty-six patients with cervical cancer and 1532 controls were eligible for study; 759 (99 percent) and 1467 (96 percent), respectively, agreed to participate. Specimens adequate for DNA assays were obtained for 747 case patients (98 percent) and 1296 controls (88 percent). The current analysis was limited to the 759 case patients and 1430 controls who reported previous sexual experience. The study participants were given a standardized interview (approximately 60 minutes long) by trained interviewers. They then had a pelvic examination, during which cells from the uterine cervix were collected for cytologic examination (controls only) and for analysis of HPV DNA. The interviewers were graduate social workers, nurses, or physicians who had received a one-week training course and participated in a pilot study.[9] Quality-control measures included interview logs, second interviews of subjects chosen at random, review of interviews, and periodic visits by staff members of Gorgas Memorial Laboratory to observe field operations at each collaborating center.

Informed Consent

All study protocols were evaluated and approved by the Gorgas Memorial Laboratory Human Subjects Committee and then by human subjects committees at each collaborating center; these committees were constituted according to the standards of the National Institutes of Health (NIH) and were approved by NIH. All study participants were volunteers who gave fully informed consent. The informed-consent documents were prepared by principal investigators (gynecologic oncologists) from each collaborating center, who made a special effort to ensure that the intended study participants would understand the documents. The forms were reviewed and modified by the NIH Office of Protection for Research Subjects.

Detection of HPV DNA Sequences

Material was collected for the assays of HPV DNA with use of a cotton-tipped swab with which the surface of the lesion (in case patients) or the cervical os (in controls) was scraped gently. The swabs were suspended in phosphate-buffered saline and stored at −20°C.

HPV DNA was detected by filter in situ hybridization.[9,10] One probe detected DNA from HPV 16/18, and a second probe detected DNA from HPV 6, HPV 11, or both (hereafter designated HPV 6/11). Cells from each sample were filtered onto three nitrocellulose papers (one for HPV 16/18, one for HPV 6/11, and one for pBR 322). DNA–DNA hybridization was carried out at 42°C in 50 percent formamide (Tm − 17°C) with a pBR 322 plasmid alone or with inserts of HPV 16 and 18 or HPV 6 and 11 DNA. The probes were labeled, with use of a commercial nick-translation system (Bethesda Research Laboratories, Gaithersburg, Md.), with [^{32}P]deoxycytidine triphosphate to a specific activity of more than 1×10^8 cpm per microgram. After hybridization, the filters were washed four times for one hour at 65°C (Tm − 15°C) and then exposed to x-ray film in a Kodak intensifying screen at −70°C for one to three days. The autoradiographs were examined independently by three observers blinded with respect to case or control status. Reactions recorded by at least two observers were used in the analysis. Reaction intensity was scored as negative (no signal), +/− (discernible signal, but insufficiently intense to define the limits of the spot clearly), 1+ (sufficiently diffuse signal to define the limits of the spot or clusters of strong signals within it), 2+ (strong signal confined to the spot), 3+ (strong signal extending beyond the limits of the spot), or 4+ (strong signal extending to the borders of adjacent spots). Specimens that reacted positively to pBR 322 (4 percent of cases and 6 percent of controls) were considered in a separate category.

Statistical Analysis

To estimate the risk of cervical cancer associated with selected exposures, we calculated odds ratios as approximations of relative risks. Multivariate logistic regression was used to adjust for potential confounding variables[11] in deriving the maximal likelihood estimates of combined relative risks and 95 percent confidence intervals. In the logistic analyses, tests for trend were performed by categorizing the exposure variable and treating the scored variable as continuous. Logistic-regression analysis was also used to test the statistical significance of multiplicative interactions. The results obtained with conditional logistic regression[12] were similar to those in the unmatched analyses that have been chosen for presentation.

Results

In order to standardize the interpretation of the filter in situ–hybridization assays, three readers interpreted the autoradiograms independently to evaluate the intensity of the reaction (Fig. 1), and they reached 85 percent agreement. To estimate the interlaboratory variation in the assay, we obtained a second cervical-swab specimen from 20 percent of the patients and controls who were enrolled between November 1986 and June 1987. The two specimens from each patient were pooled, and duplicate aliquots were assayed by Dr. L. Gissmann (Heidelberg, Germany) and ourselves. With respect to the 279 specimens, there was 76 percent agreement about the presence of HPV 16/18: there were 213 specimens for which the results were concordant, 44 in which we found DNA but Dr. Giss-

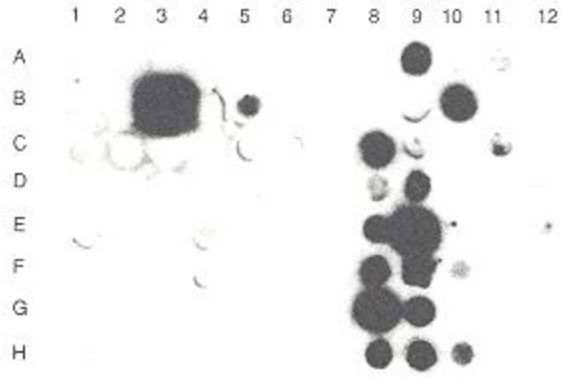

Figure 1. Autoradiogram of a Nitrocellulose Filter That Was Spotted with Cervical Samples, Processed as Described in the Methods Section, and Probed with ^{32}P-Labeled HPV 16/18 DNA.
The reactions were scored according to the intensity of the signal, on a scale from 0 to 4, defined in Methods and exemplified in the figure as follows: 0 (A-8), +/− (E-4), 1 (H-10), 2 (A-9), 3 (G-8), and 4 (B-3).

mann's laboratory did not, and 22 that we classified as negative but he classified as positive. There was 95 percent agreement in the assays of HPV 6/11. There was disagreement about 13 specimens; we coded 12 as positive that Dr. Gissmann's laboratory found to be negative or questionably positive. Other studies comparing the interlaboratory variability of this assay (or that of Southern blotting) have not yet been published.

Infection with HPV 16/18 was strongly associated with cervical cancer. Sixty-two percent of the patients but only 32 percent of the controls had detectable viral DNA (Table 1). Infection with HPV 6/11 was less strongly associated with cervical cancer (only 17 percent of the patients and 7 percent of the controls were positive).

As in other studies, associations with the risk of cervical cancer were found for the number of sexual partners (relative risk for four or more partners as

Table 1. Prevalence of HPV DNA in Patients with Cervical Cancer and Controls.*

Level of Reaction Intensity†	HPV 16/18		HPV 6/11	
	Cases	Controls	Cases	Controls
	number (percent)			
Negative	273 (37.9)	833 (68.0)	595 (82.5)	1143 (93.3)
+/−	109 (15.1)	173 (14.1)	77 (10.7)	59 (4.8)
1+	227 (31.5)	177 (14.4)	44 (6.1)	20 (1.6)
2–4+	112 (15.5)	42 (3.4)	5 (0.7)	3 (0.2)

*Twenty-six cases and 71 controls were eliminated from the analysis because of positive reactions to the pBR 322 probe. Twelve cases and 134 controls did not have samples adequate to test for DNA.

†Reaction intensity was scored as negative (no signal), +/− (discernible signal, but insufficiently intense to define the limits of the spot clearly), 1+ (sufficiently diffuse signal to define the limits of the spot or clusters of strong signals within it), 2+ (strong signal confined to the spot), 3+ (strong signal extending beyond the limits of the spot), or 4+ (strong signal extending to the borders of adjacent spots).

compared with one, 1.6; 95 percent confidence interval, 1.1 to 2.7), young age at first intercourse (relative risk for 16 years or under as compared with 20 or older, 2.1; 95 percent confidence interval, 1.5 to 2.8), no previous Pap-smear screening (relative risk, 3.0; 95 percent confidence interval, 2.3 to 3.8), and multiple live births (relative risk for eight or more deliveries as compared with three or fewer, 1.4; 95 percent confidence interval, 1.1 to 1.9). After adjustment for these factors, neither smoking, oral contraceptive use, nor limited education was a significant risk factor for cervical cancer. No relation was found between these risk factors and the presence of HPV DNA (Table 2). Although the rates of HPV 16/18 positivity varied somewhat with age, the relation was not strong. These relations were similar at all four study sites and

Table 2. Percentage of Controls with a Positive HPV 16/18 Assay According to Selected Risk Factors and Magnitude of Associated Risk of Cervical Cancer.

Risk Factors	Controls Positive for HPV 16/18*	Risk of Cervical Cancer
	%	relative risk (95% CI)†
Age (yr)		
<40	29.7	
40–49	28.5	
50–59	34.6	
≥60	39.1	
Months since Pap smear		
<48	35.5	1.00
≥48	26.4	1.64 (1.2–2.2)
Never	31.2	2.98 (2.3–3.8)
Education (yr)		
≥7	31.1	1.00
1–6	32.0	1.18 (0.9–1.6)
None	31.6	1.21 (0.8–1.8)
No. of sexual partners		
1	34.3	1.00
2	32.3	1.63 (1.3–2.1)
3	26.0	1.63 (1.2–2.2)
≥4	27.3	1.59 (1.1–2.2)
Age at first intercourse (yr)		
≥20	33.6	1.00
18 or 19	35.1	1.34 (0.9–1.8)
16 or 17	30.8	1.63 (1.2–2.2)
<16	26.4	2.07 (1.5–2.8)
No. of live births		
0–3	32.9	1.00
4–7	29.9	1.51 (1.2–2.0)
≥8	34.4	1.44 (1.1–1.9)

*The percentages of the controls testing positive were adjusted for age. Subjects without specimens or with a positive pBR test were excluded from analysis.

†Relative risks were adjusted for all factors shown, as well as for the results of the HPV 16/18 assay. 95% CI denotes confidence interval.

were not affected by the amount of HPV DNA (hybridization intensity).

The crude and adjusted relative risks of cervical cancer associated with either HPV 16/18 or HPV 6/11 were similar (Table 3). These relative risks increased significantly with the increasing intensity of the HPV 16/18 DNA hybridization reaction, from 2.1 (95 percent confidence interval, 1.6 to 2.8) in minimally detectable reactions to 9.1 (95 percent confidence interval, 6.1 to 13.6) with signal strengths of 2+ or more. These relations were similar at the four study sites and

did not differ when cases were compared with either hospital or community controls.

Although HPV 6/11 was associated less strongly with cervical cancer than HPV 16/18 and was detected less frequently overall, many subjects reacted positively to both probes. The highest risks were observed among those who reacted positively to both probes, but there was evidence of an increase in risk associated with HPV 6/11 even among subjects negative for HPV 16/18 (Table 4).

Table 4. Relative Risks of Cervical Cancer Associated with the Combined Results of DNA Hybridization Assays for HPV 16/18 and 6/11.*

Results of HPV 16/18 Assay	Results of HPV 6/11 Assay		
	Negative	+/−	1–4+
	relative risk (95 percent confidence interval)†		
Negative	1.0 [237, 791]	1.80 (0.9–3.4) [20, 29]	4.86 (2.2–10.9) [16, 13]
+/−	1.95 (1.4–2.7) [85, 163]	5.30 (2.0–14.2) [13, 7]	12.11 (3.1–47.9) [11, 3]
1–4+	5.15 (4.0–6.6) [273, 189]	6.44 (3.7–11.2.) [44, 23]	12.69 (5.0–32.0) [22, 7]

*Relative risks were adjusted for age, number of sexual partners, age at first sexual intercourse, number of live births, interval since last Pap smear, and years of education. For definitions of the levels of reaction intensity, see the footnotes to Table 1.

†Numbers in brackets are the numbers of cases and controls, respectively, in which the result was obtained.

Table 5 shows the interactions of various risk factors for cervical cancer with the detection of HPV 16/18 DNA. Although smoking and the use of oral contraceptives were not important risk factors in this study, we included them in the analysis because they have been suggested as possible cofactors with HPV in the causation of cervical cancer. There was some suggestion of an enhanced effect of HPV among women with multiple sexual partners and early age at first intercourse, but the interaction terms were not statistically significant ($P = 0.32$ and 0.09, respectively). There was no evidence of an interaction between HPV infection and oral contraceptive use.

Sixty-one of the case patients (8.2 percent) were classified histologically as having adenocarcinomas, and HPV 16/18 infection was associated with similar elevations in the relative risk for both adenocarcinomas and squamous-cell carcinomas. Finally, the same trend (and general size of relative risk) was seen for HPV 16/18 in all clinical stages of disease.

Discussion

Numerous studies have found HPV DNA in cervical cancer tissue.[3] Coupled with the ability of the virus to transform rodent cells in vitro,[13] these observations provide circumstantial evidence for the hypothesis that HPV is an etiologic agent of cervical cancer. Our large population-based case–control study examined the interrelation between the presence of HPV DNA in cervical cells and other potential risk factors for cervical cancer. We found a significant risk associated with detectable HPV 16/18 DNA; the relative risks increased from 2.1 to 9.1 with increasing intensity of the hybridization reaction and persisted after adjustment for other major risk factors.

Table 3. Relative Risks of Cervical Cancer, According to Results of DNA Hybridization for HPV 16/18 and HPV 6/11.*

Assay Results†	HPV 16/18		HPV 6/11	
	Crude Risk	Adjusted Risk (95% CI)	Crude Risk	Adjusted Risk (95% CI)
Negative	1.00	1.00	1.00	1.00
+/−	1.94	2.11 (1.6–2.8)	2.51	2.16 (1.5–3.1)
1+	3.99	4.15 (3.2–5.4)	4.24	4.57 (2.6–8.2)
2–4+	8.35	9.08 (6.1–13.6)	3.22	3.87 (0.8–17.9)

*Relative risks were adjusted for age, number of sexual partners, age at first sexual intercourse, number of live births, interval since last Pap smear, and years of education. CI denotes confidence interval.

†For definitions of the levels of reaction intensity, see the footnotes to Table 1.

Sixty-two percent of our case patients had HPV 16/18 DNA, as detected by filter in situ hybridization — a prevalence similar to the 67 percent rate reported for HPV in our earlier Latin American pilot study.[6] These rates are similar to those found in other Latin American studies with Southern blot analysis of cancer biopsy specimens: 60 percent in Panama,[14] 73 percent in Peru,[15] and 35 percent in Brazil.[16]

We also found that 32 percent of the women randomly selected as controls were infected with HPV 16/18, which is similar to the 43 percent prevalence rate in the controls in our pilot study.[6] However, the rates of detection of HPV 16/18 in the Latin American controls were higher than those reported in other large population-based surveys that have used filter in situ hybridization: 2 to 13 percent among normal women in Germany,[17] and 6 to 13 percent among women in Denmark and Greenland.[4] Part of the discrepancy may be due to the inclusion in our control sample of women with cervical abnormalities. The higher rates we observed could also represent an actual increase in the prevalence of HPV among Latin American women, who have an exceptionally high risk of cervical cancer.

Despite this high rate of background infection, the detection of HPV DNA was strongly associated with cervical cancer. Sexual behavior was also a significant risk factor for cervical cancer, but not for HPV DNA positivity — an unexpected finding. It has been tacitly assumed that most genital HPVs are venereally transmitted, but this may not be the case in all settings. Finally, the detection of HPV DNA with our assay system may not simply indicate the presence of infection but may reflect the current expression of a persistent or latent infection. The extent to which the results of various hybridization tests are affected by the form

Table 5. Relative Risks of Cervical Cancer Associated with HPV 16/18, According to Selected Risk Factors.*

Risk Factors	Hybridization Strength of HPV 16/18 DNA†		
	Negative	+/−	1–4+
	adjusted relative risk (no. of cases exposed)		
Age			
<40	1.00‡ (100)	1.28 (29)	4.08 (88)
40–49	0.98 (90)	2.13 (29)	4.51 (85)
50–59	0.78 (56)	2.52 (34)	4.72 (102)
≥60	0.75 (27)	2.11 (17)	5.63 (64)
Cigarettes smoked/day			
None	1.00‡ (187)	2.12 (75)	4.66 (227)
<10	1.03 (60)	2.17 (24)	5.91 (69)
≥10	1.02 (25)	2.10 (10)	8.00 (43)
Sexual partners (no.)			
1	1.00‡ (103)	1.91 (40)	4.72 (141)
2 or 3	1.56 (129)	3.25 (49)	8.00 (148)
≥4	1.35 (41)	3.96 (20)	8.69 (50)
Age at first intercourse			
≥18	1.00‡ (115)	1.50 (38)	4.56 (137)
16 or 17	1.35 (67)	3.87 (29)	5.53 (84)
<16	1.42 (90)	4.38 (42)	10.38 (42)
Oral contraceptives			
Never used	1.00‡ (193)	2.26 (84)	5.31 (260)
Used	1.35 (79)	2.40 (25)	6.34 (78)

*Relative risks were adjusted for age, number of sexual partners, age at first sexual intercourse, number of live births, interval since last Pap smear, and years of education.

†For definitions of the levels of reaction intensity, see the footnotes to Table 1. Column totals vary because some subjects could not respond to the questions.

‡Referent category for each risk factor shown.

of expression of the HPV genome in patients is unknown.

Interestingly, 3 percent of the case patients and 0.6 percent of the controls reacted strongly to both HPV 16/18 and HPV 6/11 probes. Positive reactions to multiple probes on filter in situ hybridization have been noted in other surveys for genital HPV.[4,17] The interpretation of these reactions requires caution, since the viral DNA of one type may cross-react with that of other types, even under stringent hybridization conditions. Thus, multiple reactivity could be the result of any of several phenomena — infection with more than one type of HPV, the presence of large quantities of DNA from a virus type to which the other probes cross-react or of an HPV variant with genomic regions that share sequence homology with the DNA probes, or lack of specificity due to insufficient stringency in the hybridization reaction. We found a high risk of cancer associated with dual reactions, and stratification revealed that a significant risk of cervical cancer was associated with HPV 6/11 among women with a negative reaction for HPV 16/18. The meaning of this is unclear, because it is difficult to infer specificity from the results of the assay used.

Filter in situ hybridization has several advantages that make it an attractive assay for use in large population studies. As compared with Southern blotting, it requires smaller amounts of material, eliminates the labor-intensive step of DNA extraction before testing, and appears to have equal sensitivity.[10,18] In situ tests do not have the same implied specificity as Southern blots, however, and the amount of cellular DNA included in the assays is not standardized. Thus, part of the difference noted between our study groups in reactivity to HPV probes may reflect variations in the DNA content of the specimens. This seems unlikely, since in a previous study we found no correlations between the number of cells in the specimen and HPV DNA positivity.[10] In addition, we found no correlation in this study between the prevalence of HPV DNA and the clinical stage (data not shown).

Other well-controlled studies should be conducted to confirm and extend these observations. Future studies should apply newly emerging laboratory methods, such as the polymerase chain reaction,[19] and should define the sensitivity and specificity of these assays carefully.[19]

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