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CASE-CONTROL STUDY OF HUMAN PAPILLOMAVIRUSES AND CERVICAL CANCER IN LATIN AMERICA

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Human papillomavirus (HPV) types 16 and 18 have been implicated as risk factors for cervical dysplasia and neoplasia. However, most studies have been observational, uncontrolled and conducted in populations at low risk for invasive cancer. We report a pilot case-control study of incident invasive cervical cancer in Panama, Costa Rica and Bogota, Colombia. Between July and September 1985 we enrolled 46 consecutive newly diagnosed invasive cervical cancer cases and 51 age-matched control women. Subjects were interviewed and samples collected for HPV DNA assays. HPV infection was defined by a filter *in situ* DNA hybidization technique under non-stringent and stringent conditions against HPV-6/11, 16 and 18 DNA probes. More cases (91%) than controls (63%) had HPV DNA detected (non-stringent) and more cases than controls had HPV-16 or 18 DNA (67% *vs.* 43%, $p = 0.02$). Age at first intercourse was the most significant risk factor for HPV 16/18 infection in all subjects. Smoking was significantly associated with cervical cancer (52% of cases *vs.* 27% controls) but was not associated with HPV infection.

Multiple types of human papillomaviruses (HPV) have been found to exist and distinct types have been associated with lesions at different anatomic sites (reviewed by Pfister, 1984). HPV-6, 11, 16 and 18 are among those types which infect the genital tract and, based on the frequent finding of HPV-16 or 18 DNA sequences in pre-malignant and malignant cervical lesions, have been implicated as risk factors for cervical neoplasia (reviewed by Gissmann and Schneider, 1986). Most studies have used the Southern blot assay to detect HPV DNA in biopsies of cervical cancers and the results have not been analyzed against those from comparable series of matched female controls. Wagner *et al.* (1984) described a non-invasive method for detecting HPV DNA sequences in cells scraped from mucosal surfaces, thus permitting easy assessment of HPV infections among normal controls as well as women with invasive cervical cancer (Schneider *et al.*, 1985). We have undertaken a case-control study of risk factors for invasive cervical cancer in Latin American women; the study includes collection of specimens to assay for HPV-6/11, 16 and 18 DNA sequences. We report here results from a pilot study in Panama, Costa Rica and Bogota, Colombia.

METHODS AND STUDY POPULATIONS

Cases

The study was conducted between July and September, 1985 in order to test study materials developed for a more detailed large-scale cervical cancer case-control study in Panama, Costa Rica, Bogota and Mexico City. We identified women with newly diagnosed invasive cervical cancer who presented at one of our study sites. Invasive cervical cancer was diagnosed and clinically staged by staff physicians from the Gynecologic Oncology Services of the 4 study sites. All diagnoses were confirmed histopathologically. Cases who had received treatment, who were older than 69 or who had not been residents of the defined study area for at least 6 months were not enrolled in the study. The study sites were the following.

Instituto Oncologico Nacional, Panama City, Panama. The Panamanian National Oncology Institute is administered by the Ministry of Health and is Panama's major cancer treatment center. Approximately 90% of all invasive cervical cancers diagnosed in Panama are referred to the Institute (Reeves *et al.*, 1984).

Hospital San Juan de Dios, San Jose, Costa Rica. This is the largest of 3 Government cancer referral centers in Costa Rica. More than 95% of invasive cervical cancers diagnosed in the country are referred to these centers for initial evaluation and San Juan de Dios attends 80% of all referrals.

Instituto Nacional de Cancerologia, Bogota, Colombia. The Colombian National Cancer Institute is the major Ministry of Health cancer referral center in Colombia. The Institute is responsible for evaluation and treatment of all medically indigent cancer patients in Bogota.

Controls

For each case, 2 age-matched (by 5-year age group) female controls were randomly selected. Controls who refused to participate were not replaced. In Panama and Costa Rica we selected one community and one hospital control and in Bogota we randomly selected both controls from hospitals.

Community controls were selected by visiting the local health center serving the patient's neighborhood, reviewing clinic census records and randomly choosing a woman within 5 years of the patient's age. The national health service systems are such that virtually all residents of a neighborhood are registered at their local health center. If a control was found to have had a previous diagnosis of cancer or a hysterectomy she was replaced by another randomly selected woman. Current cervical pathology was not considered in control selection.

Hospital controls were selected in Panama from inpatient services of the hospital which had referred the patient; in Costa Rica from San Juan de Dios Hospital; in Bogota from one of 4 tertiary level government hospitals. Hospital controls were randomly selected from women admitted with non-gynecologic conditions; women with a previous diagnosis of cancer, who had undergone hysterectomy or who were admitted with endocrine or smoking-related diseases were not eligible as controls. However, current (previously undiagnosed) cervical pathology was not considered in control selection.

Although the study protocol specified 2 age-matched controls for each case, this study reports data from 46 cases and 51 controls. Because of time, logistic and other constraints we terminated the pilot study before all eligible controls were identified and contacted. Since hospital and community con-

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trols were similar with respect to most variables they were combined in the analyses.

Data collection

Interviews

All study protocols were reviewed by Human Subjects Committees at the collaborating institutions as well as by the Gorgas Memorial Laboratory Human Subjects Committee and the Office of Protection of Research Subjects, National Institutes of Health, US. All subjects were volunteers who gave informed consent to participation in the study.

Study subjects participated in standardized, structured interviews which were conducted by specially trained interviewers and required approximately 1 hr to administer. Completed interviews were reviewed and then coded for computer data processing.

All eligible cases presenting in Costa Rica and Bogota were successfully recruited into the study and only one of 20 cases identified in Panama could not be interviewed, due to language problems. Age distribution and clinical stage of cases from the 3 sites were identical. No hospital controls from Bogota or Costa Rica refused to participate in the study and only one hospital control from Panama refused to participate. Eighty-one percent of selected community controls from Costa Rica and 64% from Panama were successfully enrolled into the study and those who were not selected for enrollment were missed because they resided too far from the capital city.

Specimens

Samples of cells were collected from the surface of cervical lesions of cases and the cervical os of controls. We used cotton-tipped swabs which were subsequently suspended in 4 ml of phosphate-buffered saline and stored at −20°C until tested.

Detection of HPV DNA sequences

HPV DNA sequences were detected by a filter *in situ* DNA hybridization technique (Grunstein and Hogness, 1975). Briefly, the specimens were thawed and the cells pelleted by centrifugation at 1,000 *g* for 10 min. Cell pellets were resuspended in 400 μl of a buffer containing 5 μg/ml denatured salmon sperm DNA in 6 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). The cells were then filtered onto nitrocellulose paper which had been presoaked in 6 × SSC and 100 μl of each specimen were filtered onto 3 separate nitrocellulose papers. Cell lysis and DNA denaturation were effected by 2 5-min treatments in 1 M NaCl and 0.5 M NaOH. The filters were then neutralized by 2 5-min treatments in 1.5 M NaCl and 0.5 M Tris (pH 7.4). After air-drying the specimens were digested with 20 ml of a solution containing 2 mg/ml proteinase K, 0.01 M Tris (pH 7.8), 0.05 M EDTA (pH 8.0) and 0.05% sodium dodecyl sulfate (SDS). The filters were washed 3 times in chloroform and once in 2 × SSC, then heated at 80°C for 90 min.

DNA-DNA hybridization was carried out as described by Thomas (1980). The filters were incubated at 42°C for 2 hr in a pre-hybridization buffer containing 50% formamide, 5 × SSC 50 mM sodium phosphate (pH 6.5), 250 μg/ml denatured salmon sperm DNA and 5 × Denhardt's solution. Hybridization was carried out at 42°C for 12 to 15 hr in a buffer consisting of 4 parts pre-hybridization buffer and one part 50% (w/v) dextran sulfate. Recombinant plasmids containing pBR322 and HPV-16, HPV-18, HPV-6C or HPV-11 DNA were kindly provided by Dr. L. Gissmann (DKFZ, Heidelberg). Plasmid DNAs were labelled by nick translation (Rigby *et al.*, 1977) to a specific activity of more than 1 × 10^8 cpm/μg using a ^{32}P dCTP and a commercial nick translation system (Bethesda Research, Gaithersburg, MD). The labelled DNAs were denatured at 100°C for 5 min, cooled and added to the hybridization solution at a final concentration of 1 × 10^5 cpm/ml. Filters were reacted with HPV-16 DNA, HPV-18 DNA or a mixture of HPV-6C and HPV-11 DNA. After hybridization, the filters were washed 4 times for 1 hr at 42°C (non-stringent conditions) in 2 × SSC containing 0.1% SDS. They were then exposed at −70°C for 1 to 3 days to X-ray film using a Kodak intensifying screen. The filters were subsequently washed 4 more times at 68°C (stringent conditions) in 2 × SSC containing 0.1% SDS followed by 2 5-min washes at room temperature in 0.1 × SSC containing 0.1% SDS after which they were again autoradiographed.

TABLE I – HUMAN PAPILLOMAVIRUS DNA IN MATERIAL FROM CASES AND CONTROLS, CERVICAL CANCER CASE CONTROL STUDY 1985 – PANAMA, COSTA RICA AND BOGOTA

	Case n = 46		Control n = 51	
	Number + DNA	% +	Number +	% +
Any HPV[1]	42	91%	32	63%
Panama	16/19	84%	9/17	53%
Costa Rica	13/13	100%	14/20	70%
Bogota	13/14	93%	9/14	64%
HPV-16/18[2]	31	67%	22	43%
Panama	7	37%	3	18%
Costa Rica	12	92%	10	50%
Bogota	12	86%	9	64%
HPV-6/11	9	20%	7	14%
Panama	7	37%	4	24%
Costa Rica	1	8%	3	15%
Bogota	1	7%	0	00%

[1]Any HPV refers to HPV DNA detected in any of the 3 non-stringent assays (HPV-16, HPV-18, HPV-6/11). Cases vs. controls: Chi-square 9.4 $p = 0.002$; Odds ratio 6.2; 95% CI 1.8–24.2. [2]HPV-16/18 refers to specimens positive in stringent assay against either type irrespective of infection with HPV-6/11. Cases vs. controls: Chi-square 4.8 $p = 0.03$; Odds ratio 2.7; 95% CI 1.1–6.8. HPV-6/11 refers to specimens positive against HPV-6/11 in stringent assays but negative against HPV-16/18 in stringent assay.

To confirm specificity, the filters were dehybridized by incubation for 1 hr at 65°C in a solution containing 5 mM Tris (pH 8.0), 0.2 mM EDTA, 0.05% sodium pyrophosphate and 0.5 × Denhardt's solution as described by Thomas (1980). The filters were then pre-hybridized, hybridized under stringent conditions using radiolabelled pBR322 plasmid DNA and subjected to autoradiography. Finally, the filters were again dehybridized and reprobed under stringent conditions using a radiolabelled mixture of HPV-16 and HPV-18 DNAs which had been excised from recombinant plasmids, gel-separated and affinity-column-purified.

All X-ray films were examined independently by 3 observers and those specimens recorded as positive by 2 or more observers were scored as positive. Rehybridization with pBR322 DNA was positive in 7 of the originally selected subjects and these were excluded from all analyses. Rehybridization with HPV-16/18 DNA excised from the plasmid revealed a 92% agreement between the initial assay using plasmid DNA and the repeat assay using excised viral DNA. Most of the disagreement consisted of samples positive in the initial assay but negative on reanalysis and this probably represented loss of signal on repeat dehybridization. The specimens from women residing in different geographic areas were tested simultaneously.

Statistical analysis

We used the relative risk as approximated by the odds ratio to measure associations and evaluate effects of exposure factors. Statistical analyses were done with standard microcomputer systems and software. Statistical comparisons of pro-

TABLE II – DISTRIBUTION OF CERVICAL CANCER RISK FACTORS AMONG CASES AND CONTROLS, CERVICAL CANCER CASE-CONTROL STUDY 1985—PANAMA, COSTA RICA AND BOGOTA

Attribute[1]	Prevalence of attribute among		Odds ratio
	Cases	Controls	
History of smoking[3]	24/46 (52)[2]	14/51 (27)	2.88
First intercourse at 16 years or younger[4]	24/45 (53)	17/50 (34)	2.22
Two or more sexual partners[5]	28/44 (64)	24/51 (47)	1.97
No formal education	10/46 (22)	8/51 (16)	1.49
History of Pap smear	26/45 (58)	34/49 (69)	0.60

[1]Denominators may vary depending on unknown responses.–[2]Number with attribute/number responding to interview question (% with attribute).–[3]Fisher's exact test, $p = 0.01$.–[4]Fisher's exact test, $p = 0.05$.–[5]Fisher's exact test, $p = 0.08$.

portions utilized the Chi-square test of homogeneity for RxC tables, the Chi-square test for trend or Fisher's exact test. Stepwise logistic regression analysis was used to determine the combined importance of risk factors identified in univariate analyses (Cox, 1970; Engleman, 1985).

RESULTS

The distribution of reactivity with the viral DNA probes is shown in Table I. Reactivity under non-stringent hybridization conditions was observed in 42 of 46 (91%) women with cervical cancer and 32 of 51 (63%) control women (Chi-square = 9.4, $p = 0.002$). The percent positive for DNA from any HPV type did not differ significantly between cases (84 to 100%) or controls (53 to 70%) from different study areas. Under stringent hybridization conditions, HPV-16 or HPV-18 DNA (HPV-16/18) was significantly more frequent in cases (31 of 46, 67%) than in controls (22 of 51, 43%) (Chi-square = 4.8, $p = 0.03$). Reactivity with HPV-16/18 DNA accounted for most of the positivity observed under non-stringent hybridization conditions among cases and controls from Costa Rica and Bogota. However, only 37% of cases and 18% of controls from Panama reacted with HPV-16/18 DNA probes under stringent conditions (the remainder of positives were HPV-6/11). Interestingly, HPV-16/18 DNA sequences were detected in 19 of 35 (54%) specimens obtained from hospital controls but from only 3 of 16 (19%) specimens obtained from community controls.

The distributions of risk factors and reactivity of specimens with HPV DNA probes were analyzed by geographic area. Since the relationships were similar in all areas, the data were pooled for presentation. Cancer cases differed from controls with respect to variables commonly associated with cervical cancer (Table II). Fifty-three percent of cases gave a history of having had sexual intercourse by 16 years of age while only 34% of control women gave such a history. Multiple sexual partners were claimed by 64% of cancer cases compared to 47% of control women while a history of cigarette smoking was obtained from 52% of cases and 27% of controls. A slightly greater percentage of cases than controls had received no formal education and of those receiving formal education only 22% of cases achieved a secondary grade level as compared to 28% of control women. A history of prior Pap smear was obtained less frequently from cases (58%) than from controls (69%).

The distribution of HPV-16/18-related DNA (stringent conditions) among the study subjects according to age is shown in Table III. Among cases, the positivity rates were similar in all age groups but among control women the rates increased with increasing age. Thus, the highest risk (odds ratio = 5.8) was observed among the youngest women, being nearly 3 times that observed among the oldest age group.

The results of stepwise logistic regression analysis of case-control differences, with no interaction terms, are shown in Table IV. These analyses were based on 88 of 97 subjects (91%) for whom complete data were available. We entered factors which were significant or approached significance in univariate analyses into the model: smoking history (ever *vs.* never), HPV infection (HPV-16/18 *vs.* any or no HPV), age at first intercourse (16 or younger *vs.* older than 16), number of life-time sexual partners (1, 2, 3 or more). When age was forced into the model as a continuous variable, only infection with HPV-16/18 and age at first intercourse of 16 years or less entered as risk factors (goodness of fit Chi-square = 107.7, $p = 0.017$; Chi-square = 104.31, $p = 0.025$, respectively). There was no evidence of 2-way interactions between age at first intercourse and infection with HPV-16 or -18.

DISCUSSION

Durst *et al.* (1983) have shown, using the Southern blot technique, HPV DNA sequences in a high proportion of preinvasive and invasive cervical lesions. They found HPV-16 DNA sequences in 11 of 18 (61%) cancers from German women but in only 8 of 23 (35%) cancers from women in Kenya or Brazil. HPV-18 DNA was subsequently cloned and this probe detected DNA sequences in 9 of 36 (25%) cancer specimens from Kenya, Uganda or Brazil (Boshart *et al.*, 1984). A number of subsequent reports concerning invasive cervical cancer biopsies analyzed by Southern blots have confirmed the occurrence of HPV-16- or HPV-18-related DNA. Most studies involved small numbers of patients from low-risk areas so that prevalence of HPV infection varied greatly. For example, HPV-16 DNA was detected in 18% of cases from Minnesota (3/17)

TABLE III – HPV-16/18 DNA IN MATERIAL FROM CASES AND CONTROLS, CERVICAL CANCER CASE-CONTROL STUDY 1985—PANAMA, COSTA RICA AND BOGOTA[1]

	Age[2]		
	< 30	30–49	50+
Case	5/8 62%	9/16 56%	15/20 75%
Control	2/9 22%	7/16 44%	13/23 56%
Odds ratio	5.8	1.6	2.3

[1]Results are expressed as number of specimens with HPV DNA/number in subgroup. HPV DNA is reported as positive if HPV-16 or HPV-18 DNA was detected under stringent conditions (irrespective of evidence for other HPVs).–[2]Age was unknown for 5 subjects.

TABLE IV – ODDS RATIO ESTIMATES FROM LOGISTIC REGRESSION ANALYSIS OF CERVICAL CANCER CASES AND CONTROLS, PANAMA, COSTA RICA AND BOGOTA—1985

	Odds ratio	95% C.I.
HPV-16/18[1]	2.67	1.07–6.70
Age at first intercourse[2]	2.36	0.96–5.80

[1]HPV-16/18 is reported as positive if HPV-16 or HPV-18 DNA was detected under stringent conditions *vs.* negative for HPV-16/18 (irrespective of evidence for other HPVs).–[2]Age at first intercourse was dichotomized as 16 or younger *vs.* older than 16.

(Fukushima *et al.*, 1985), in 45% of cases from East Anglia (5/11) (Scholl *et al.*, 1985), 46% from Italy (6/13) (DiLuca *et al.*, 1986), 65% from Panama (13/20) (Prakash *et al.*, 1985), 92% from London (12/18) (McCance *et al.*, 1985), 13 of 13 cases from Peru (Lancaster *et al.*, 1986); in addition, a similar variation in positivity rates for HPV-16 or 18 has been reported by Japanese investigators (Tsunokawa *et al.*, 1986; Yoshikawa *et al.*, 1985; Tomita *et al.*, 1986).

None of these studies included rigorously selected controls so that the strength of association between HPV and invasive cervical cancer has not been defined. Published data on HPV infection of normal women have documented variable rates of infection (possibly due to small sample sizes and different sampling frames). For example, HPV-16 DNA could not be found in biopsies of normal cervical tissue from 15 German women (Boshart *et al.*, 1984), 2 Italian women (DiLuca *et al.*, 1986), 12 women from East Anglia (Scholl *et al.*, 1985), 10 Japanese women with cervical squamous metaplasia (Tomita *et al.*, 1986), or 17 Panamanian women with cervicitis (Prakash *et al.*, 1985). Other studies found HPV-16 DNA in 11% (12 of 104; 1 of 9) (Toon *et al.*, 1986; Macnab *et al.*, 1986), 18% (3 of 17) (McCance *et al.*, 1985), and 38% (5 of 13) (Cox *et al.*, 1986) of histopathologically normal biopsy specimens from women with non-cancerous conditions.

In this study we utilized filter *in situ* hybridization to define HPV infection in a series of incident cervical cancer cases and age-matched controls. This method is superior to Southern blotting for testing large numbers of specimens, and filter *in situ* also has the advantage that it does not require biopsy and thus facilitates sampling of normal controls. Our results confirmed a significant association between HPV-16 or HPV-18 DNA (detected under stringent hybridization conditions) and invasive cervical cancer. The overall odds ratio was 2.7 with 95% confidence limits between 1.1–6.8. We also found variation in the prevalence of HPV-16/18 infection in cervical cancer cases from different areas but the association of HPV infection with cervical cancer was similar in all 3 study sites. This observation supports the concept of geographic variation between HPV-16/18 and cervical cancer suggested by earlier studies. Small sample size and limitations of the technique do not allow meaningful speculation as to the prevalence of other HPV types.

An unexpectedly high proportion of control women were infected with HPV. Fifty percent or more of the specimens from matched control women reacted with one or more HPV probes under non-stringent hybridization conditions and, under stringent conditions, 43% of control women had HPV-16 or 18 DNA sequences detected. Although these rates are high in comparison to those described by other authors, there are several reasons to believe that they accurately reflect HPV infection in normal women from the 3 study areas. First, as noted above, HPV infection rates varied in both cases and controls from the 3 areas. Second, several studies have indicated that other sexually transmitted viral agents are more prevalent in populations with high cervical cancer rates; HPV infection in our study was similar to HSV-2 antibody prevalence in Latin American women (Rawls *et al.*, 1986). Third, HPV-16/18 prevalence differed between hospital (54%) and community controls (19%) (the age distributions of hospital and community controls were equivalent). Evidence of higher rates of infection with venereally transmitted herpes simplex virus type 2 among hospital controls as compared to community controls was observed by Priden and Lilienfeld (1971). Finally, the major risk factor for HPV infection in controls appeared to be age (rates varied from 22% in controls younger than 30 to 56% in those aged 50 or over) and for the most part our study population was composed of older women. The low rates we observed in younger women are similar to those seen in the study of McCance *et al.* (1985) who found HPV-16 DNA in biopsies of 17% of control women and also to a recent study of HPV-16/18 infection in pregnant women (Schneider *et al.*, 1986); the majority of these women were presumably under 30 years of age and 16% had HPV DNA.

In addition to HPV-16/18 infection, cases and controls differed significantly with respect to behavioral attributes classically associated with invasive cervical cancer (Rotkin, 1973) and the frequency of these attributes among populations has been shown to vary and correlate with cancer incidence (Rawls *et al.*, 1986). We used logistic regression analyses to assess the combined importance of HPV-16/18 infection and behavioral risk factors. Since age was the only effect modifier of HPV infection detected in this study, we forced age (as a continuous variable) into the model. Although numbers were small, infection with HPV-16/18 was the most important risk factor, followed by age at first intercourse; neither smoking nor number of life-time sexual partners contributed significantly to risk for cervical cancer in this pilot study.

A disadvantage of the filter *in situ* hybridization technique used in this study is the inability to confirm specificity of the reaction since the size of the putative DNA is not determined, as is possible in the Southern blot method. False positives may arise if DNA sequences related to the plasmid carrying the viral DNA are present in secretions or biopsy material (Ambinder *et al.*, 1986; Diegutis *et al.*, 1986). Indeed, we found that 7 specimens reacted with radiolabelled pBR322 DNA and these were excluded from analysis. Ninety-two percent of the positive reactions could be reconfirmed by reprobing under stringent conditions with purified HPV-16 DNA excised from the pBR322 plasmids. In a separate study we used Southern blotting to assay biopsies from the Panamanian invasive cervical cancer cases and 14 of the 16 (88%) cancer patients with positive HPV-16/18 filter *in situ* hybridizations (stringent conditions) were also positive by the other method (Caussy *et al.*, in press). Thus, it is unlikely that the high positivity rate observed is due to false-positive reactions related to hybridizations with pBR322 DNA sequences.

Our data suggest high prevalence rates of HPV-16/18-related viruses in the Latin American populations studied. Age-specific prevalence rates observed among control women were compatible with those of other viruses that establish persistent infections and thus accumulate with age. The data also suggest that women with cervical cancer are infected with HPV-16 or 18 at an earlier age than women without cancer. However, the age-specific pattern observed in our study could also represent a cohort effect. The data presented support an etiological role of HPV in cervical cancer; however, further delineation of the natural history of genital HPV infections and of the interactions of HPV infections with other risk factors of cervical cancer is needed before this issue can be clarified.

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