Antibodies to Human Papillomavirus 16 and Subsequent *in Situ* or Invasive Cancer of the Cervix[1]

Keerti V. Shah,[2] Raphael P. Viscidi, Anthony J. Alberg, Kathy J. Helzlsouer, and George W. Comstock

Departments of Molecular Microbiology and Immunology [K. V. S.] and Epidemiology [A. J. A., K. J. H., G. W. C.], Johns Hopkins School of Public Health, and Department of Pediatrics, Johns Hopkins School of Medicine [R. P. V.], Baltimore, Maryland 21205

Abstract

Our objective was to examine whether past infection with human papillomavirus (HPV)-16, as determined by an antibody assay, is a risk factor for subsequent cervical cancer. Incident cases of *in situ* or invasive cervical cancer occurring between 1975 and 1990 in a cohort of over 11,000 healthy women in Washington County, MD, were identified. The baseline sera of cases and of matched controls, collected in 1974, were examined for IgG antibodies reactive with virus-like particles of HPV-16, a cancer-associated HPV, and HPV-6, a low-risk HPV. Postdiagnosis sera of 11 cases were also assessed similarly. Fourteen cases of invasive and 28 cases of *in situ* cervical cancer and 83 matched controls were evaluated. The main outcome measure was the risk of cervical cancer in women who had HPV-16 or HPV-6 antibodies in prediagnostic sera. Antibodies to HPV-16 but not to HPV-6 were a marker for subsequent occurrence of cervical cancer. Case sera were reactive more often and more strongly with HPV-16 virus-like particles than were sera of matched controls. The presence of antibodies to HPV-16 was significantly associated with an increased risk of cervical cancer (odds ratio, 3.9; 95% confidence limits, 1.4, 10.7); high antibody levels to HPV-16 were associated with an even greater risk of cervical cancer (odds ratio = 7.5, 95% confidence limits 1.5, 36.3). The association with cervical cancer was strengthened after adjustment for smoking and years of education. In tests of 11 pairs of pre- and postdiagnostic sera, HPV-16 antibodies did not decline markedly over a 7–13-year time period, and seroconversion to HPV-16 appeared to have occurred in 2 cases. The serological data indicate that HPV-16 infection is associated with future risk of cervical cancer and strengthen the evidence for the etiological role of HPVs in cervical cancer.

Introduction

The evidence linking HPV[3] infections of the genital tract with invasive cervical cancer rests on detection of HPVs in cancers and cancer precursors (1–4), on the identification of molecular mechanisms by which HPVs act as carcinogens (5, 6), and on prospective studies in which cervical HPV infections are shown to be a risk factor for low- and high-grade squamous intraepithelial lesions of the cervix (1, 7). However, evidence demonstrating a temporal relationship between past HPV infections and subsequent development of cervical cancers has been difficult to obtain, both because reliable serological assays for the presence of HPV antibodies have only been developed recently (8, 9) and because prediagnostic serum samples of women who develop cervical cancer are rarely available.

In 1996, Lehtinen *et al.* (10) reported the results of a nested case-control study in which it was shown that the presence of antibodies to HPV-16 capsids was associated with an increased risk of subsequent cervical cancer in Finnish women. We conducted a similar study in Washington County, MD, where serum specimens from a large number of healthy residents who donated blood specimens in 1974 were stored to allow subsequent identification of early serological markers of cancers and of other diseases (11). Prediagnostic sera from incident cases of *in situ* or invasive cervical cancer and matched controls among volunteer donors were assayed for antibodies reactive with VLPs of HPV-16 and HPV-6. These two serotypes were selected because HPV-16 has the strongest association and HPV-6 one of the weakest associations with invasive cervical cancer: HPV-16 is recovered from about 50% and HPV-6 from less than 0.5% of cervical cancer tissues (3). We report here that antibodies to HPV-16 are early markers for the future development of cervical cancer, whereas those to HPV-6 are not.

Materials and Methods

Serum Specimens. In the autumn of 1974, a community-wide campaign was conducted to collect 15 ml of blood from adult volunteers in Washington County in western Maryland. A few more specimens were collected in the summer of 1975 during the course of a private census of the county. Serum was stored at −70°C.

At the time blood was collected, a brief history was taken. The information included birth date, race, sex, marital status, years of schooling completed (as a measure of socioeconomic status), and cigarette smoking. Of the total 25,820 specimens

Received 8/8/96; revised 11/19/96; accepted 1/6/97.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This study was supported in part by United States Public Health Service Grant CA56514 (to R. P. V.), Research Grant DAMD 17-94-J-4265 from the Department of the Army (to K. J. H.), and Research Career Award HL21670 from the National Heart, Lung and Blood Institute (to G. W. C.).
[2] To whom requests for reprints should be addressed, at The Johns Hopkins University, School of Public Health, Department of Molecular Microbiology and Immunology, 615 North Wolfe Street, Baltimore, MD 21205.

[3] The abbreviations used are: HPV, human papillomavirus; VLP, virus-like particle; CL, confidence limit.

collected, 11,009 were donated by females older than 18 years who were identified as Washington County residents in a private census conducted in the summer of 1975. They made up approximately one-third of the adult female population of the county. Participation was better among women ages 35–64 years and those with more education.

Among this donor population, 50 cases of cervical cancer, either *in situ* or invasive, were identified from the Washington County cancer register as having been diagnosed for the first time between January 1, 1975, and June 1, 1990. The ratio of observed numbers of invasive cases in this donor population to the number expected from the age- and race-specific rates published by the Surveillance, Epidemiology and End Results registers for 1978–1981 (12) was 1.05. Reporting of *in situ* cases is known to be incomplete because only the cases diagnosed at Washington County Hospital, the one general hospital in the county, were recorded in the Washington County cancer register during the period of this study.

In an earlier study of cervical cancer in this population, cases were compared to controls with respect to serum micronutrients (11). For each case, two controls were identified from blood donors who were not in the cancer register and were not known to have died at the time the case was diagnosed. Although controls were not individually followed for the development of cervical cancer, the application of sex- and age-specific incidence rates from the Surveillance, Epidemiology and End Results registries for 1984–1988 to the control population indicated that less than one case of invasive carcinoma is likely to have developed among them during the study period (12). Controls had to be within 1 year of age of the case, one control being younger and the other older. In addition, cases and controls were matched with respect to race, date of blood collection (within 1 month), time of blood drawing in relation to last meal (within 2 h), and time since last menstrual period.

Sera remaining after aliquots had been prepared for the above study were used for the present study. Of the 50 cases, sera were available for 42 case-control sets. These sets included 14 cases of invasive cervical carcinoma and 2 matched controls per case, 27 cases of carcinoma *in situ* with 2 controls per case, and 1 case of carcinoma *in situ*, for which only 1 matched control was available. Prediagnostic sera from cases and controls were frozen and thawed twice. Aliquots were sent to the laboratory in containers with dry ice and were assayed in sets; a set consisted of sera from the case and from the matched controls. Postdiagnostic specimens were available for 11 cases. They had been thawed only once, for this study.

Sera were assayed as sets, with nine quality control specimens inserted at irregular intervals. The identities of the various serum specimens were not revealed to the laboratory until the assays had been recorded and reported. Coefficients of variation were calculated for each quality control set by dividing the standard deviation by its mean and were expressed in percentages.

Assays for Antibodies. Antibodies were measured in ELISA using HPV-16 and HPV-6 VLPs. Recombinant baculoviruses expressing the L1 and L2 proteins of HPV-16 and the L1 protein of HPV-6b (gifts of J. T. Schiller, National Cancer Institute, Bethesda, MD) were used to prepare the respective VLPs by infection of sf9 insect cells. VLPs were purified by cesium chloride density gradient centrifugation as described by Kirnbauer *et al.* (13). The purified VLP preparation (50 ng) in 50 μl of PBS, pH 7.2, was added to the wells of a 96-well polystyrene microtiter plate (Corning-Costar, Cambridge, MA; catalogue number 25802), which was incubated overnight at 4°C and then washed five times. These and subsequent washes were done with PBS-0.05% Tween 20. Serum specimens were tested in duplicate wells at a 1:10 dilution in PBS-0.05% nonfat milk. After incubation with sera for 1 h at 37°C, the plate was washed five times, and bound immunoglobulin was detected with horseradish peroxidase-conjugated recombinant protein G (Zymed Laboratories, San Francisco, CA). Peroxidase substrate (ABTS and hydrogen peroxide solution, Kirkegaard and Perry, Gaithersburg, MD) was incubated at room temperature for 30 min and absorbance was read in a microtiter plate reader (Molecular Devices Corp., Menlo Park, CA) at 405 nm. For each serum specimen, the difference in absorbance was calculated by subtracting the mean absorbance obtained in wells coated only with buffer from the mean absorbance obtained in wells coated with antigen. A preassigned cutoff point for seropositivity (mean absorbance plus 3 SDs, excluding the outliers) was established from the reactivity of 57 reference sera obtained from college women at low risk of prior HPV infection (14). These women reported no or only one lifetime sexual partner, had no cytological abnormalities by Pap smear testing, and were negative by L1 consensus primer PCR for HPV DNA in the genital tract. An absorbance value of 200 or greater for HPV-16 was considered "positive" and indicative of a past infection, and a value of 400 or greater was considered "strongly positive" and indicative of high antibody levels. A value of 600 or greater for HPV-6 was regarded as positive and indicative of a previous infection.[4]

Statistical Analyses. The statistical significance of differences between median absorbance values of cases and controls was determined by the Mann-Whitney *U* test. The ranking of cases with respect to the controls in each set was assessed by a nonparametric test described by Kwa *et al.* (15). Odds ratios and their CLs were calculated by the Mantel-Haenszel method as outlined by Schlesselman (16) and adjusted for smoking history and years of education by conditional logistic regression (17).

Results

The characteristics of cases and controls are shown in Table 1. One case was African-American and the rest were white, the distribution reflecting the fact that there are very few nonwhites in Washington County. Cases and controls were similar with respect to age and marital status. Cases had an average of 10.9 years of schooling and controls 11.3 years. Cases were more likely to currently smoke, largely because a much smaller proportion of cases than controls had quit smoking. The number of *in situ* cases diagnosed and registered decreased markedly with time, reflecting the increasing proportion of *in situ* cases diagnosed outside the hospital and consequently not recorded in our cancer register. There was no demonstrable time trend for invasive cases.

There was some variation in absorbance values of the nine sets of quality control sera; the median coefficients of variation were 26.0% for HPV-16 and 14.4% for HPV-6. The distributions of absorbance values due to HPV-16 and HPV-6 antibodies are summarized in Fig. 1 for case and control sera. The absorbance values of the two matched controls in each set were averaged for this analysis. The median values and interquartile ranges for HPV-16 were 103 (47–288) and 74 (46–114) for cases and controls, respectively, a difference that approached statistical significance ($P < 0.06$). The highest HPV-16 absorb-

[4] R. P. Viscidi and K. V. Shah, unpublished data.

Table 1 Frequency and percentage distribution of selected characteristics among cervical cancer cases and controls, Washington County, MD, 1974

Characteristic	No.		%	
	Cases (n = 42)	Controls (n = 83)	Cases (100.0)	Controls (100.0)
Age (years)				
≤24	9	18	21.4	21.7
25–34	12	24	28.6	28.9
35–44	6	10	14.3	12.1
45–54	8	17	19.1	20.5
55–64	4	8	9.5	9.6
65–74	3	6	7.1	7.2
Marital status				
Never married	7	16	16.7	19.3
Previously married	8	14	19.1	16.9
Married	27	53	64.3	63.9
Education (yrs)				
≤8	7	9	16.7	10.8
9–11	15	21	35.7	25.3
12	15	40	35.7	48.2
13+	5	13	11.9	15.7
Cigarette smoking				
Never	19	46	45.2	55.4
Past	1	12	2.4	14.5
Current	22	25	52.4	30.1
Diagnosis				
Carcinoma *in situ*	28	NA[a]	66.7	NA
Invasive	14		33.3	
Period of diagnosis				
1975–77	14	NA[a]	33.3	NA
1978–80	9		21.4	
1981–83	10		23.8	
1984–86	4		9.5	
1987–90	5		11.9	

[a] NA, not applicable to controls.

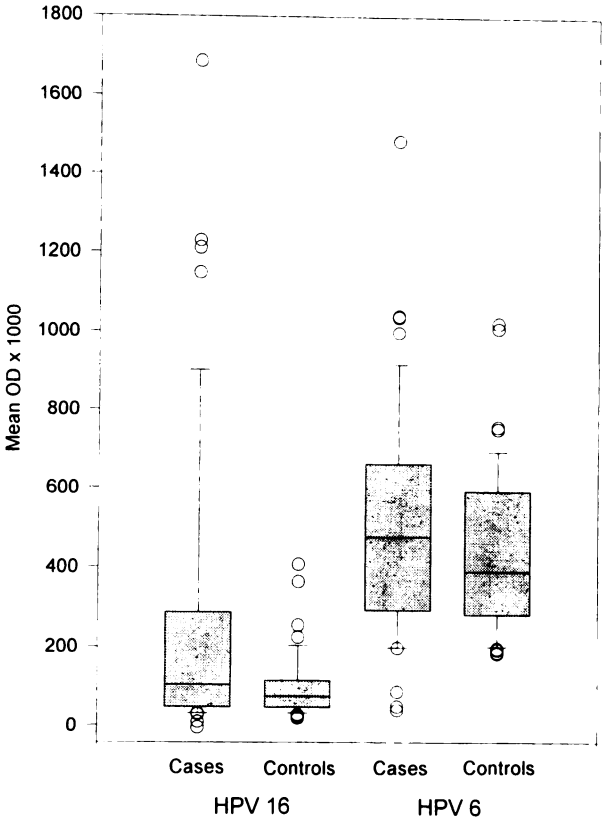

Fig. 1. Prediagnostic antibody levels against HPV-16 and HPV-6 among cervical cancer cases and matched controls. Serum specimens from 42 cases of *in situ* or invasive cervical cancer and 83 matched controls were tested by ELISA for antibody to HPV-16 and HPV-6 VLPs. The ELISA absorbance values are displayed in box plots. *Box*, 25th–75th percentile; *horizontal solid line* in the box, median value; *bar*, 90th percentile; O, outlier values.

ance value in the 42 sets of control sera was 406; 9 of the 42 case sera had absorbance values greater than 406, ranging from 409 to 1686. For HPV-6, the median values and interquartile ranges for cases and controls were 476 (292–663) and 390 (281–594), respectively, a difference that was not statistically significant (P = 0.48).

The absorbance value for each case was also compared to the individual absorbance of the matched controls. It was found (Table 2) that 55% of the cases had values of HPV-16 that were higher than either of the controls, and only 19% of the cases had values lower than both controls. In contrast to this highly significant difference (P = 0.003), the ranking of cases and controls did not differ significantly with respect to absorbance values for HPV-6.

The above results indicate a qualitative difference between cases and controls. To examine the magnitude of these differences, the difference between the case serum absorbance and the average absorbance of the control sera was calculated for each case-control set. The HPV-16 absorbance value was higher for the case serum than the average of the two matched controls in 26 of the 42 (62%) case-control sets. Furthermore, the largest case-control differences tended to occur in the sets in which the case serum had the higher value (Wilcoxon sign rank test P value = 0.01). For example, the case serum absorbance value was higher in 13 of 14 serum sets in which the case-control differences in absorbance values were 120 or greater. For HPV-6 antibodies, the case-control differences in absorbance values were not statistically significant (Wilcoxon sign rank test P value, 0.24).

Table 2 Rank of cervical cancer cases relative to matched controls with respect to absorbance values for HPV-16 and HPV-6

Rank of case in matched triplets[a]	HPV-16	HPV-6
Highest	23	19
Middle	10	10
Lowest	8	12
P value	0.003	0.14

[a] In the one matched pair, the case had higher optical values than the matched control for HPV-16 and HPV-6.

For HPV-16, 33.3% (14 of 42) of the cases were positive at baseline compared to only 12.0% (10 of 83) of the controls; 19% (8 of 42) of the case sera and 4% (3 of 83) of the control sera were strongly positive. Cases and controls were more nearly alike with respect to HPV-6; although 33.3% (14 of 42) of the cases were positive, 25.3% (21 of 83) of the controls were also positive. The difference between cases and controls with respect to HPV-16 was statistically significant (P = 0.01 for both positive and strongly positive), whereas the difference with respect to HPV-6 was not (P = 0.45). Matched odds ratios estimated by the Mantel-Haenszel method indicated an increased risk of cervical cancer of 3.9-fold for women positive for HPV-16 antibodies at baseline, 7.5-fold for women strongly

Table 3 Risk of developing cervical cancer according to antibody levels against HPV-16 and HPV-6, cigarette smoking, and years of education at study baseline in 1974

Characteristic	No. (cases/controls)	Odds ratio[a]	95% CLs
HPV-16[b]			
Negative	28/73	1.0	Referent
Positive	14/10	3.9	1.4, 10.7
HPV-16[c]			
Negative	34/80	1.0	Referent
Positive	8/3	7.5	1.5, 36.3
HPV-6[d]			
Negative	28/62	1.0	Referent
Positive	14/21	1.4	0.7, 3.1
Cigarette smoking			
Never	19/46	1.0	Referent
Past	1/12	0.2	0.03, 1.8
Current	22/25	2.3	1.0, 5.1
Education (yrs)			
≤8	7/9	1.0	Referent
9–11	15/21	0.7	0.2, 3.3
12	15/40	0.3	0.07, 1.4
≥13	5/13	0.3	0.05, 1.7

[a] Odds ratios adjusted for the matched variables age, race, date of blood collection, hours since last meal, and time since last menstrual period.
[b] Positive is defined as absorbance ≥ 200.
[c] Positive is defined as absorbance ≥ 400.
[d] Positive is defined as absorbance ≥ 600.

positive for HPV-16 antibodies at baseline, and 1.4-fold for those positive for HPV-6 antibodies at baseline (Table 3). After adjustment for history of cigarette smoking and years of education, the association between HPV-16 antibodies and cervical cancer risk was strengthened; the odds ratio increased from 3.9 (Table 3) to 5.5 (95% CL, 1.6, 19) for HPV-16 absorbance ≥200, and from 7.5 (Table 3) to 14 (95% CL, 2, 97), for HPV-16 absorbance ≥400. Analyses stratified by HPV-16 antibodies and by never and ever smoked, although limited by small numbers, showed that HPV-16 antibodies were associated with increased risk in both smokers and nonsmokers. In contrast, after adjustment, the association between HPV-6 antibodies and cervical cancer remained essentially the same. Women who were current smokers in 1974 were at increased risk of developing cervical cancer, whereas the controls were more likely than the cases to have quit smoking (Table 3). Women who had completed more than 8 years of school were at reduced risk of cervical cancer relative to women who had completed 8 years or less (Table 3). Neither of these associations was materially altered after adjustment for HPV-16 and HPV-6 antibodies.

We examined whether the results could have been affected by the possibility that the cases diagnosed soon after recruitment were prevalent cases at the time of recruitment. The data were analyzed limited to case-control sets in which the case was diagnosed over 2 years after the study began (n = 31 sets). The results of these analyses were nearly identical to the results presented in Table 3. We also evaluated whether the observed associations differed according to whether the case was diagnosed with *in situ* or invasive cervical cancer; the same overall pattern of associations held true in both groups (data not shown).

Postdiagnostic specimens available from 11 cases were tested for HPV-16 antibodies (Table 4). Eight of these sera were obtained within 1 year of diagnosis and three were collected 5–9 years after diagnosis. The age at diagnosis ranged from 22 to 82 years, and the time interval between the paired specimens was 6–13 years. Three patterns were evident in the absorbance values of the paired sera. In four cases, both pre- and postdiagnostic specimens were negative for HPV-16 antibodies. In five cases, the first serum was positive, and except in one of these cases (case 9), the antibody levels in the second serum, taken 7–13 years after the first serum, were not markedly different from those in the first serum. In two instances, the first serum was negative but the second was positive, suggesting that HPV-16 infection may have occurred after the baseline prediagnostic sera were obtained.

Discussion

The availability of over 11,000 sera from healthy women in Washington County, MD, allowed us to examine whether antibodies to HPV-16 VLPs were predictive of subsequent cervical cancer. Prediagnostic sera were available for 42 women who developed *in situ* or invasive cancer 1–16 years after the serum collection in 1974. We examined the prediagnostic sera from these 42 women and from 83 matched controls, in ELISA, for antibodies to VLPs of HPV-16, the HPV type most consistently associated with cervical cancer. We also tested, in parallel, the same sera with VLPs of HPV-6, a HPV type that is almost never found in cervical cancers.

We compared the absorbance values of cases and controls. In all analyses, antibodies to HPV-16 VLPs appeared to be markers for subsequent cervical cancer. They were detected in 33% of the prediagnostic sera of cases as compared to 12% of the sera of controls. Sera were strongly positive with HPV-16

Table 4 HPV-16 antibodies in pre- and postdiagnostic sera from 11 cases of cervical cancer

Category	Case	Age at diagnosis	Years between bleedings	Time of second bleeding[a]	HPV-16 absorbance values	
					Prediagnosis	Postdiagnosis
Both sera negative	1	43	6	<1	84	167
	2	32	8	<1	90	169
	3	57	8	5	74	119
	4	40	10	9	142	108
First serum positive	5	70	7	<1	219	173
	6	82	13	<1	880	606
	7	43	8	1	294	242
	8	56	8	1	1,453	1,431
	9	47	13	8	573	198
Seroconversion	10	22	7	<1	60	214
	11	59	9	1	130	342

[a] Years after diagnosis

VLPs in 19% of the cases but only in 4% (3 of 83) of the controls. The risk of cervical cancer increased 4-fold in women who tested positive for antibodies to HPV-16 and 7-fold in women who tested strongly positive; the risks were greater after adjustment for smoking and education. Consistent with previous studies, being a current cigarette smoker was associated with an increased risk of cervical cancer (18), and educational attainment was inversely associated with the risk of cervical cancer (19). In contrast to the results with HPV-16 VLPs, antibodies to HPV-6 VLPs were not associated with an increased risk of subsequent cervical cancer.

Although the presence of HPV-16 VLP antibodies was predictive of cervical cancer, these antibodies were detected in only one-third of the case sera. This finding was not unexpected. The HPV-16 VLP assay detects antibodies that are largely specific for HPV-16 infection. Only about one-half of the cervical cancers are associated with HPV-16, and only about one-half of the individuals who have documented HPV-16 infection develop antibodies to HPV-16 VLPs (9). Also, as demonstrated by the examination of pre- and postdiagnostic sera, some infections may have occurred *after* 1974, when the prediagnostic sera were collected.

Our results confirm the data from a similar study of Finnish women, in which antibodies to HPV-16 VLPs were reported in 25% of the cases and 2% of the controls (10). In addition, the data from the paired samples of cases in our study showed that antibodies tended to persist for many years. The similarity of the findings among Finnish women and among women in Washington County provides added assurance of a causal role of HPV-16 in the pathogenesis of cervical cancer.

References

1. IARC. Monograph on the Evaluation of Carcinogenic Risks to Humans. Vol. 64: Human Papillomaviruses. Lyon, France: IARC, 1995.

2. Muñoz, N., Bosch, F. X., deSanjosé, S., Tafur, L., Izarzugaza, I., Gili, M., Viladiu, P., Navarro, C., Martos, C., Ascunce, N., Gonzalez, L. C., Kaldor, J. M., Guerrero, E., Lörincz, A., Santamaria, M., Alonso de Ruiz, P., Aristizabal, N., and Shah, K. The causal link between human papillomavirus and invasive cervical cancer: a population-based case-control study in Colombia and Spain. Int. J. Cancer, *52:* 743–749, 1992.

3. Bosch, F. X., Manos, M. M., Muñoz, N., Sherman, M., Jansen, A. M., Peto, J., Schiffman, M. H., Moreno, V., Kurman, R., Shah, K. V., and the International Biological Study on Cervical Cancer Study Group. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J. Natl. Cancer Inst., *87:* 796–802, 1995.

4. Schiffman, M. H., Bauer, H. M., Hoover, R. N., Glass, A. G., Cadell, D. M., Rush, B. B., Scott, D. R., Sherman, M. E., Kurman, R. J., Wacholder, S., Stanton, C. K., and Manos, M. M. Epidemiologic evidence showing that HPV infection causes most cervical intraepithelial neoplasia. J. Natl. Cancer Inst., *85:* 958–964, 1993.

5. zur Hausen, H. Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. *In:* H. zur Hausen (ed.), Human Pathogenic Papillomaviruses, pp. 131–156. Heidelberg: Springer-Verlag, 1994.

6. Shah, K. V., and Howley, P. M. Papillomaviruses. *In:* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (eds.), Virology, Ed. 3, pp. 2077–2109. Philadelphia, PA: Lippincott-Raven Publ., 1996.

7. Koutsky, L. A., Holmes, K. K., Critchlow, C. W., Stevens, C. E., Paavonen, J., Bechmann, A. M., DeRouen, T. A., Galloway, D. A., Vernon, D., and Kiviat, N. B. A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation to papillomavirus infection. N. Engl. J. Med., *327:* 1272–1278, 1992.

8. Kirnbauer, R., Hubbert, N. L., Wheeler, C. M., Becker, T. M., Lowy, D. R., and Schiller, J. T. A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. J. Natl. Cancer Inst., *86:* 494–499, 1994.

9. Schiller, J. T., and Roden, R. B. S. Papillomavirus-like particles. Papillomavirus Rep., *6:* 121–128, 1995.

10. Lehtinen, M., Dillner, J., Knekt, P., Luostarinen, T., Aromaa, A., Kirnbauer, R., Koskela, P., Paavonen, J., Peto, R., Schiller, J. T., and Hakama, M. Serologically diagnosed infection with human papillomavirus type 16 and risk for subsequent development of cervical carcinoma: nested case-control study. Br. Med. J., *312:* 537–539, 1996.

11. Batieha, A. M., Armenian, H. K., Norkus, E. P., Morris, J. S., Spate, V. E., and Comstock, G. W. Serum micronutrients and the subsequent risk of cervical cancer in a population-based nested case-control study. Cancer Epidemiol., Biomarkers & Prev., *2:* 335–339, 1993.

12. Ries, L. A., Hankey, B. F., Miller, B. A., Hartman, A. M., and Edwards, B. K. Cancer Statistics Review 1973–88. NIH Publication No. 91-2789. Bethesda, MD: National Cancer Institute, 1991.

13. Kirnbauer, R., Taub, J., Greenstone, H., Roden, R., Durst, M., Gissmann, L., Lowy, D. R., and Schiller, J. T. Efficient self-assembly of human papillomavirus type 16 L1 and L1–L2 into virus-like particles. J. Virol., *67:* 6929–6936, 1993.

14. Viscidi, R. P., Kotloff, K. L., Clayman, B., Russ, K., Shapiro, S., and Shah, K. V. Prevalence of antibodies to HPV type 16 virus-like particles in relation to cervical HPV infection among college women. Clin. Diagn. Lab. Immunol., *4:* 122–126, 1997.

15. Kwa, H. G., Cleton, F., Wang, D. Y., Bulbrook, R. D., Bulstrode, J. C., Hayward, J. L., Millis, R. R., and Cuzick, J. A prospective study of plasma prolactin levels and subsequent risk of breast cancer. Int. J. Cancer., *28:* 673–676, 1981.

16. Schlesselman, J. J. Case-Control Studies. Design, Conduct, Analysis, pp. 213–216. New York: Oxford University Press, 1982.

17. Breslow, N. E., and Day, N. E. Statistical Methods in Cancer Research, Vol. 1. The Analysis of Case-Control Studies, pp. 248–278. Lyon, France: IARC, 1980.

18. Winkelstein, W. Smoking and cervical cancer–current status: a review. Am. J. Epidemiol., *131:* 945–957, 1990.

19. Daly, M. B., Bookman, M. A., and Lerman, C. E. Female reproductive tract: cervix, endometrium, and ovary. *In:* P. Greenwald, B. S. Kramer, and D. L. Weed (eds.), Cancer Prevention and Control, pp 585–610. New York: Marcel Dekker, 1995.

Antibodies to human papillomavirus 16 and subsequent in situ or invasive cancer of the cervix.

K V Shah, R P Viscidi, A J Alberg, et al.

Cancer Epidemiol Biomarkers Prev 1997;6:233-237.