Detection of Human Papillomavirus DNA in Cytologically Normal Women and Subsequent Cervical Squamous Intraepithelial Lesions

Kai-Li Liaw, Andrew G. Glass, M. Michele Manos, Catherine E. Greer, David R. Scott, Mark Sherman, Robert D. Burk, Robert J. Kurman, Sholom Wacholder, Brenda B. Rush, Diane M. Cadell, Patti Lawler, David Tabor, Mark Schiffman

Background: **Human papillomavirus (HPV) infection has been strongly associated with cervical carcinoma and its cytologic precursors, squamous intraepithelial lesions (SIL). We investigated the risk of SIL prospectively following polymerase chain reaction (PCR)-based DNA testing for a wide range of genital HPV types in a cohort of initially cytologically normal women, to clarify the role of HPV in the etiology of SIL.** ***Methods:*** **Starting in April 1989, 17 654 women who were receiving routine cytologic screening at Kaiser Permanente (Portland, OR) were followed for the development of incident SIL. During follow-up, 380 incident case patients and 1037 matched control subjects were eligible for this nested case–control study. Cervical lavages collected at enrollment and, later, at the time of case diagnosis (or the corresponding time for selection of control subjects) were tested for HPV DNA using a PCR-based method. The data were analyzed as contingency tables with two-sided *P* values or, for multivariable analyses, using odds ratios (ORs) with 95% confidence intervals (CIs).** ***Results:*** **In comparison with initially HPV-negative women, women who tested positive for HPV DNA at enrollment were 3.8 times (95% CI = 2.6–5.5) more likely to have low-grade SIL subsequently diagnosed for the first time during follow-up and 12.7 times more likely (95% CI = 6.2–25.9) to develop high-grade SIL. At the time of diagnosis, the cross-sectional association of HPV DNA and SIL was extremely strong (OR = 44.4 and 95% CI = 24.2–81.5 for low-grade SIL and OR = 67.1 and 95% CI = 19.3–233.7 for high-grade SIL). HPV16 was the virus type most predictive of SIL, even low-grade SIL.** ***Conclusions:*** **These findings are consistent with the hypothesis that HPV infection is the primary cause of cervical neoplasia. Furthermore, they support HPV vaccine research to prevent cervical cancer and efforts to develop HPV DNA diagnostic tests. [J Natl Cancer Inst 1999;91:954–60]**

Human papillomavirus (HPV) infection, measured by DNA testing of exfoliated cervical cells, has been strongly and consistently associated with cervical carcinoma *(1)* and its cytologic precursors, squamous intraepithelial lesions (SIL) *(2)*. Based on all available laboratory and epidemiologic evidence, the association is considered causal *(3)*. Epidemiologic evidence for an etiologic role for HPV has come mainly from cross-sectional studies and prevalent case–control investigations [reviewed in *(3)*].

The few prospective studies [reviewed in *(3)*] addressing whether HPV infection predicts new SIL have been supportive but limited. Reliable, sensitive HPV testing methods, such as MY09*/MY11 consensus primer polymerase chain reaction (PCR) *(4,5)* and GP5+/ GP6+ general primer PCR *(6)*, which type the wide range of genital HPVs, have only been well standardized in the past few years *(7)*. By necessity, earlier studies used HPV testing methods that had limited sensitivity or detected only a few cancer-associated types of HPV (mainly, type 16) *(8)*.

With increased standardization of HPV DNA testing methods in the 1990s, reliable large-scale prospective data are now emerging *(9,10)*. However, we still lack reliable estimates of the risk of incident SIL following cervical HPV infection for the many genital HPV types now known. A very large study of thousands of women is required to examine individual HPV types in relation to subsequent risk of SIL because a new diagnosis of SIL occurs in only a few percentage of women per year.

Accordingly, to investigate the etiologic role of HPV infection prospectively in the early pathogenesis of cervical carcinoma, we undertook this incident case–control study. The study was nested into a 5-year follow-up of 17 654 initially cytologically normal women at Kaiser Permanente (Portland, OR). Cervical cells collected from study subjects at enrollment (when all women were cytologically normal) were tested for the presence of HPV DNA by a sensitive PCR-based method. The results permitted us to examine the prospective role of different HPV types in predicting the full spectrum of cervical SIL that developed in the large cohort population. By taking another HPV test at case diagnosis (or the comparable time at control selection), we were also able to estimate risks of SIL related to concurrent HPV detection, as well as to combinations of HPV positivity at enrollment and diagnosis.

Subjects and Methods

Study Population

Between April 1989 and November 1990, 23 702 women aged 16 years or older, obtaining a routine Pap smear at one of seven Kaiser Permanente gynecology or health appraisal clinics in Portland, OR, were recruited for a natural history study of HPV and cervical neoplasia. More than 95% of eligible women agreed to participate. At that time, Kaiser Permanente was providing cytologic screening to a demographically representative one quarter of adult women in Portland (mainly middle-class, white women), and the study clinics were performing about half the Pap smears performed by the health maintenance organization.

To examine risk factors for new (presumably first ever) diagnosis of SIL, an incidence cohort was formed. Women with a medical history of hysterectomy or cervical SIL/cancer were excluded by review of Kaiser Permanente surgery and pathology records and by a self-administered questionnaire returned by about 60% of the women. Women with SIL diagnosed at the enrollment examination were studied in a separate prevalent case–control study *(2)*. The remaining 17 654 women formed the incidence cohort, designed to represent the general population of women with no history or current evidence of SIL *(11)*.

Enrollment Examination

Conventional one-slide Pap smears, obtained with the use of endocervical brushes and ectocervical Ayre spatulas, were used to prepare the slides in

Affiliations of authors: K.-L. Liaw, S. Wacholder, M. Schiffman, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD; A. G. Glass, D. R. Scott, B. B. Rush, P. Lawler, Kaiser Permanente, Portland, OR; M. M. Manos, C. E. Greer, Cetus Corporation, Emeryville, CA; M. Sherman, R. J. Kurman, Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD; R. D. Burk, Albert Einstein College of Medicine, Bronx, NY; D. M. Cadell, D. Tabor, Westat Inc., Rockville, MD.

Correspondence to: Mark Schiffman, M.D., M.P.H., National Institutes of Health, Executive Plaza South, Rm. 7066, Bethesda, MD 20892.

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accordance with the standard screening practice in these clinics. A descriptive cytologic classification scheme that predated the Bethesda System *(12)* was used by the Kaiser cytopathology laboratory at that time. Of the 14 possible diagnoses, only women with diagnoses of "normal" or "benign reactive atypia" were included in the incidence cohort, because these correspond to the negative screening diagnoses in the Bethesda System, i.e., "within normal limits" and "benign cellular changes."

Following the Pap smear, a 10-mL saline lavage of the cervix *(13)* was collected from all participants. Of the 10-mL cervical lavage, a 1-mL unprocessed aliquot was frozen at −70 °C for PCR-based HPV DNA testing.

Follow-up of the Incidence Cohort

By definition, the 17 654 women included in the incidence cohort had normal cytologic diagnoses at enrollment. Accordingly, they were followed passively (without any intervention to encourage their return) by reviewing the computer-accessible records of their voluntary, routine Pap smear examinations from enrollment until the end of December 1994. During that time, Kaiser Permanente was recommending yearly Pap smears, and many cohort members complied. The mean frequency of Pap smears during follow-up was 0.6 per woman per year, with 22% of the women having none. The youngest and oldest cohort members were the most likely to be lost to follow-up.

Identification of Possible Case Patients

All Pap smears were initially diagnosed with the use of the local classification that we combined into Bethesda System terminology. A possible incident case was identified when a Pap smear result indicated possible SIL, including a new diagnosis of "severe reactive changes, possible dysplasia," "dysplasia" (including "mild," "moderate," and "severe"), or "carcinoma" during follow-up. Incident carcinoma was not expected and only one possibly invasive case (included as high-grade SIL in the analysis) was observed in this generally low-risk and well-screened cohort, despite the large number of subjects.

Because microscopic evidence of SIL can be transient and diagnostically subtle, a number of criteria were applied to study only incident, confirmed case patients with SIL (including equivocal SIL, called "atypical squamous cells of undetermined significance" or ASCUS in the Bethesda System) in comparison to valid control subjects. The major steps in case–control confirmation are outlined in Fig. 1 and referred to in the sections below.

Initially, 770 possible case patients were enumerated during follow-up. However, Kaiser Permanente has a policy of a minimal 9-month gap between two routine Pap smear examinations; thus, the 99 possible case patients who were diagnosed within the first 9 months subsequent to enrollment were not approached because they probably represented prevalent abnormalities (Fig. 1). In addition, we chose not to investigate the common and ambiguous transition from the Kaiser Permanente diagnoses of "benign reactive atypia" at enrollment to "severe reactive atypia, possible dysplasia" at follow-up (n = 101; Fig. 1) because misclassification between these two pathologically adjacent, slightly abnormal categories was likely *(14)*. As a result of this choice, the proportion of ASCUS and low-grade case patients with benign reactive atypia (rather than a totally negative diagnosis) at enrollment was conservatively biased to be small. The few instances were down-graded case patients, resulting from pathology review (*see below*) of initially more severe classification of incident SIL diagnoses.

After these initial exclusions, a total of 570 probable case patients remained (Fig. 1). Probable case patients were met at their confirmatory colposcopy appointment for collection of a repeat cervical lavage. The participation rate at the colposcopy visit was lowered by many women who chose to have colposcopy performed outside Kaiser Permanente. A telephone interview following the appointment was conducted by a trained interviewer to elicit more detailed information than the enrollment questionnaire on demographic characteristics, Pap smear history, and reproductive and sexual histories, by use of a formal, previously validated epidemiologic questionnaire.

Control Subject Selection

Up to three control subjects were matched to each probable case patient on two main variables: age (±5

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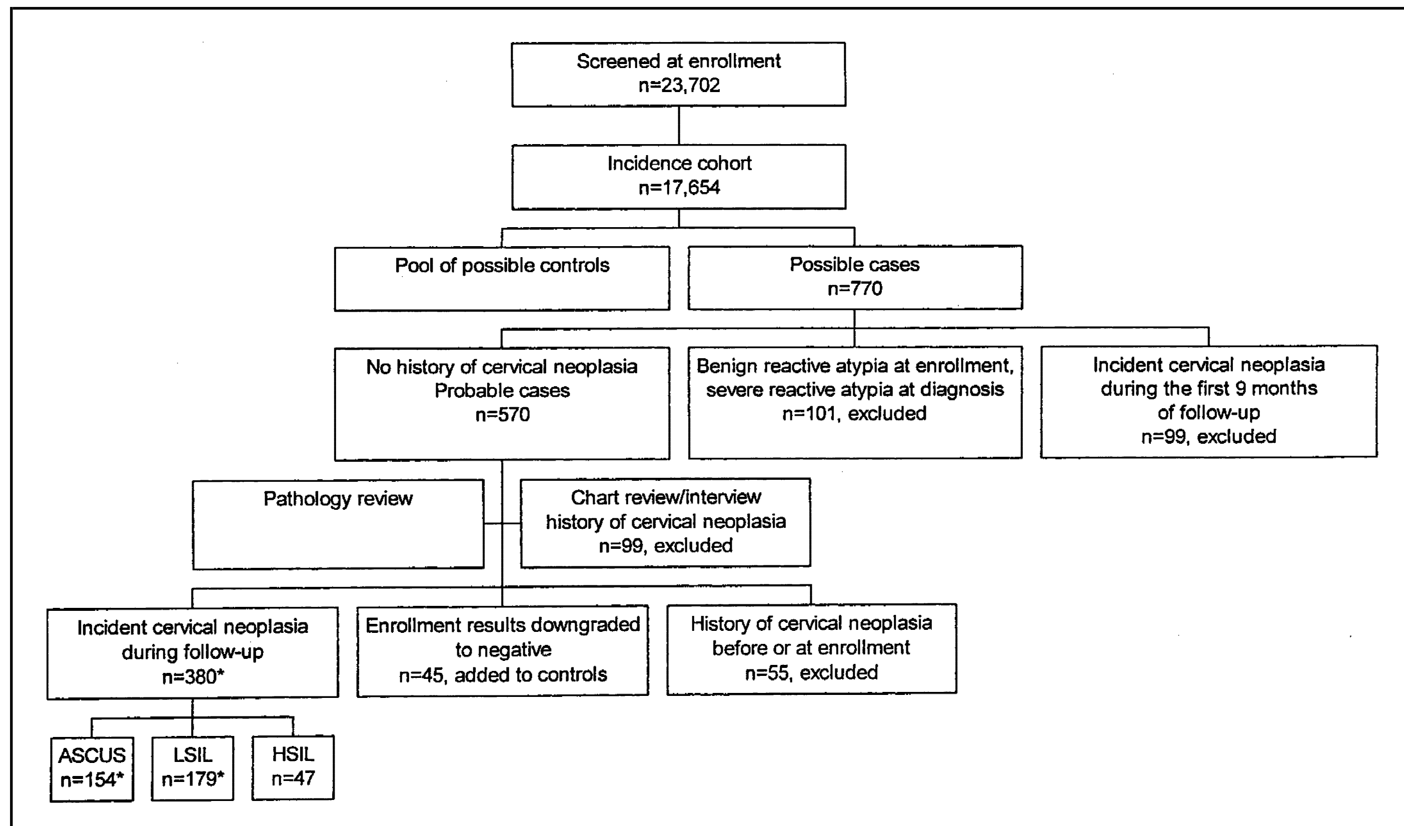

Fig. 1. To study incident cases of squamous intraepithelial lesions (SIL), we attempted to exclude individuals with past or current diagnoses of SIL or related cervical treatments, using review of relevant computer files and patient charts, a follow-up interview to the enrollment questionnaire, and strict pathology review of all available cervical cytology and histology slides. The flow of exclusions leading to the final analytic group of case patients is shown. *Final case patients included nine from the original control group; seven patients with incident atypical squamous cells of undetermined significance (ASCUS) and two patients with incident low-grade SIL were discovered on pathology review. LSIL = low-grade SIL; HSIL = high-grade SIL

years) and follow-up time (0–60 days longer than the index case patient to ensure that control subjects had at least as much time to develop SIL). We also chose to match on three “nuisance” variables, which proved however to be only weak confounding influences: enrollment clinic (gynecology versus lower-risk health appraisal clinics performing only routine screening), enrollment cytology diagnosis (normal or benign reactive atypia), and return of the enrollment questionnaire.

At identification of a probable case, a listing of upcoming appointments of possibly eligible matching control subjects from the incidence cohort was generated by computer linkage with enrollment files. For most case patients who did not have unusual matching factors, there were many possible control subjects. To prevent a potential selection bias, we did not contact possible control subjects in any way to encourage their follow-up screening (because case patients had not been contacted prior to completing their diagnostic appointments). When a possible control subject did arrive and undergo follow-up screening, she was approached for collection of a cervical lavage (96% participation). As for the case patients, telephone interviews were also used to collect epidemiologic information. All participants provided full, written informed consent. All key study documents and protocols were approved by the local Institutional Review Board of Kaiser Permanente.

Confirmation of Probable Case Patients and Control Subjects

At enrollment, members of the incidence cohort had been checked for medical history of SIL/cancer by an examination of relevant computer files and by a self-administered questionnaire. To minimize further the possibility of inclusion of patients with prevalent or recurrent cervical neoplasia in this study of incident SIL, the hard-copy medical charts were retrieved and reviewed by an experienced medical abstractor for all probable case patients and control subjects. The women found to have histories of SIL that had been missed at enrollment were excluded. In addition, a very few women whose medical history of cervical neoplasia came to light only during the telephone interview were excluded from the analysis. These procedures led to the exclusion of 99 probable case patients (Fig. 1) and 19 control subjects.

Finally, all stored Pap smears and biopsy slides of probable case patients and control subjects before and after enrollment (up to diagnosis) were reviewed. In addition to the enrollment and diagnostic smears, a median of three cervical smears per subject was reviewed, averaging one intervening smear during follow-up (interquartile range, 0–2).

Our review of smears and biopsy specimens used the Bethesda System, with final diagnoses divided into the classifications of normal, ASCUS, low-grade SIL, and high-grade SIL. Pap smears were screened by a single cytotechnologist who referred all but unequivocally normal smears, along with all histology slides, to a single pathologist (D. R. Scott). Subsequently, all possibly abnormal smears and all histology slides were reviewed masked to prior diagnoses by a second pathologist (M. E. Sherman). After a comparison of the two pathologists’ reviews, all Pap smears and histology slides with discrepant readings were evaluated by a third pathologist (R. J. Kurman), again masked to previous diagnoses. The final case–control classification was made with the use of a simple algorithm by the National Cancer Institute principal investigator (M. Schiffman) based on all review results. Almost all diagnoses were achieved either by an agreement between the first two reviewers or by a simple majority. The handful of remaining case patients was adjudicated by taking the middle ground of the dissenting diagnoses (e.g., normal versus ASCUS versus low-grade SIL equals ASCUS). For case patients who had both a biopsy and a diagnostic cytologic result (confirmed by the reviewers), the more severe diagnosis was used for the data analysis.

The pathologists’ review excluded 55 probable case patients because their Pap smears at or prior to enrollment were reviewed as suggesting SIL (Fig. 1), and 45 probable case patients were excluded because their diagnostic Pap smears were downgraded to negative on review (Fig. 1). The latter excluded case patients were included as control subjects in this study.

Thus, a total of 371 (65.1%) of 570 probable case patients were confirmed as truly incident case patients after the rigorous record review, interview, and pathology review. Moreover, among the reviewed control subjects, nine were found to be missed incident case patients because their diagnostic slides indicated low-grade SIL ($n = 2$) or cytologically confirmed ASCUS ($n = 7$). They were included, therefore, as case patients, raising the final total of confirmed case patients to 380.

The confirmed case patients included 154 with a final diagnosis of ASCUS, 179 with low-grade SIL, and 47 with high-grade SIL. Thirty-nine (83%) of the high-grade SIL case patients were histologically confirmed, to be what would typically be termed cervical intraepithelial neoplasia (CIN) 2 or 3. Many case patients with low-grade SIL did not have a biopsy performed; thus, only 70 (39%) of low-grade SIL diagnoses were confirmed despite rigorously reviewed cytology. Given that the diagnosis of ASCUS here required at least suspicion of SIL by more than one pathologist, the term should be viewed as more specific than a typical community cytologic diagnosis of ASCUS.

Smears from the 1056 control subjects were virtually all confirmed as cytologically normal based on their available smears. Only 55 control subjects were excluded because of prevalent SIL at enrollment or prior to enrollment. Nine more were reclassified as incident case patients (*see above*). After inclusion of the 45 additional control subjects who were originally diagnosed as possible incident case patients but down-graded subsequent to the pathology review, there were 1037 confirmed control subjects.

Detection of HPV DNA

Cervical samples collected at enrollment and diagnosis from a subject were assayed for HPV DNA by use of the same PCR-based method. The first third of the specimens was completed at Cetus Corporation (Emeryville, CA). The remaining specimens were tested by use of the same protocol several years later when the study was completed, in two large batches at the Albert Einstein College of Medicine (Bronx, NY). The laboratories performing PCR were masked to any information regarding the subjects.

All available specimens from case patients and control subjects collected at enrollment and diagnosis were tested by use of PCR, except 21 samples (19 control subjects, one ASCUS case patient, and one low-grade SIL case patient) collected at enrollment and four samples (four control subjects) at diagnosis, which had insufficient material.

PCR-Based Assay at the First Laboratory

Aliquots of cervical lavage were amplified by the L1 consensus primer pair MY09* and MY11, as described previously *(4,5)*. Amplification products were hybridized with a generic HPV probe mixture to determine positivity and with type-specific oligonucleotide probes to identify individual HPV types. The probes included 6/11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 68, 73, PAP155, PAP291, and W13B. Because the probes for HPV6 and HPV11 were originally mixed at Cetus, detection of these two types was not differentiated and was labeled HPV6/11; however, retesting showed that type 6 predominated. Amplification of a human β-globin gene fragment was used as an internal control for sample integrity. Positive controls (SiHa cervical cancer cell lines) and negative controls (K562 human DNA cell line) were interspersed to ensure validity. In addition, at Cetus, a masked repeat aliquot was inserted as every 20th specimen with near-perfect reliability.

PCR-Based Assay at the Second Laboratory

The PCR detection and typing of HPV DNA at Albert Einstein are described in detail in previous publications *(7,15–17)*. Thirty microliters of sedimented cellular material from the lavage was digested by incubation in 100 μL of K buffer with 200 μg/mL of proteinase K. A 10-μL aliquot of this material was then amplified with the MY09*/MY11 L1 consensus primers *(4,5)*. Ten microliters of the PCR reaction mix was analyzed by agarose gel electrophoresis and transferred to nylon filters. The filters were hybridized overnight with [α-^{32}P]deoxycytidine triphosphate-labeled generic probes for HPV and an oligonucleotide for β-globin.

PCR products that were positive with the HPV generic probe mix were analyzed for multiple HPV types, including HPV types 2, 6, 11, 13, 16, 18, 26, 31, 32, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 72, 73 (PAP238A), AE2, AE5, AE6, AE7, AE8, W13B, PAP291, and PAP155. Specimens that were positive by the generic probe mix but negative by all type-specific probes were considered to represent undetermined HPV types. Thus, in aggregate, 17 additional types, namely, types 2, 13, 32, 34, 61, 62, 64, 67, 69, 70, 72, 74, AE2, AE5, AE6, AE7, and AE8, were assayed during the second testing phase *(7)*.

Statistical Methods

HPV detection (overall and type specific) at enrollment and diagnosis was treated as a binary variable (positive versus negative) for calculation of risk estimates. Because the number of subjects infected with a specific type of HPV was often too small to be assessed individually with reliability, the different types of HPV were grouped according to their association with invasive cervical carcinoma *(1)*.

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The most important type, HPV16, was maintained as a separate category, because it is common and found in about half of cervical carcinoma case patients worldwide *(1).* For grouped analyses, HPV types found in at least 1% of cervical carcinoma patients, including types 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, were categorized as the "other cancer-associated types." The remaining types that were tested for in this study were categorized as "low-risk types." Subjects infected with multiple types of HPV were classified hierarchically into the highest HPV risk group applicable (16 > other cancer-associated types > low-risk types).

Specimens displaying very weak hybridization signals with the generic HPV probe only (40 from enrollment and 37 from diagnosis) were excluded from all HPV-related statistical analyses to minimize confusion due to their possible false positivity. Specimens of "undetermined type" (*see above*) were categorized as HPV positive; however, these were excluded in type-specific analyses. In analyses categorizing HPV types as cancer associated or low risk, the specimens of undetermined types were categorized as low risk.

The data were analyzed by use of standard contingency table methods, with significance testing when performed as two-sided. To estimate relative risks, we calculated odds ratios (ORs) and 95% confidence intervals (CIs) associating HPV infection with risk of SIL using multiple logistic regression models. The matching variables and other possible confounding variables including age (16–19 years, 20–29 years, 30–39 years, and ≥40 years), follow-up time (<790 and ≥790 days), enrollment clinic, enrollment cytology diagnosis, return of enrollment questionnaire, and number of visits between enrollment and diagnosis were included in unconditional regression models for adjustment. Unconditional modeling was used despite the matched case–control design, because the exclusion during clinical–pathologic review of unconfirmed case patients and control subjects prevented the efficient maintenance of the individual matching. However, conditional logistic regression models yielded similar results, even though they were necessarily restricted to case–control strata still containing at least one case and one control following review *(18).*

The interlaboratory agreement on HPV DNA detection between Cetus and Albert Einstein College of Medicine was shown in a previous methodologic comparison *(19)* to be good (91%); however, testing laboratory was retained as a variable in the statistical models to prevent any statistical laboratory effect in this much larger series.

RESULTS

Despite the many reviews and exclusion of case patients and control subjects found to be invalid, the distribution of the nuisance variables remained basically similar between all case patients and all control subjects (*P*>>.05 for all comparisons by standard chi-square testing). However, case patients tended to be younger (*P* = .003). Also, the length of follow-up was longer in the case patient group (*P* = .007) because of typically brief follow-up of the many probable case patients who were excluded from analysis when found on review to be prevalent. When case patients were divided into subgroups according to their SIL severity, the similarity to the overall control subject group was not maintained. Most strikingly, compared with the entire group of control subjects, ASCUS patients tended to be older, while low-grade SIL and high-grade SIL patients were younger.

As shown in Table 1, compared with the initially HPV-negative women, those who tested positive for HPV DNA at enrollment were at a fourfold risk of developing incident ASCUS (OR = 3.8; 95% CI = 2.5–5.8), a fourfold risk of developing low-grade SIL (OR = 3.8; 95% CI = 2.6–5.5), and a 13-fold risk of developing high-grade SIL (OR = 12.7; 95% CI = 6.2–25.9). Analysis by HPV type groups (Table 1) showed that women who had HPV16 infection at enrollment were at an even higher risk of developing high-grade SIL than those who had other types of HPV.

While the percentage of HPV-positive control subjects remained the same at enrollment and diagnosis, it was not necessarily the same women infected at both

Table 1. Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for cervical neoplasia associated with human papillomavirus (HPV) at study enrollment and at diagnosis*,†

	No. of control subjects	Patients with ASCUS			Patients with low-grade SIL			Patients with high-grade SIL		
		No.	OR	95% CI	No.	OR	95% CI	No.	OR	95% CI
HPV at enrollment										
Negative‡	834	100	1	—	100	1	—	13	1	—
Positive‡	157	50	3.8	2.5–5.8	73	3.8	2.6–5.5	29	12.7	6.2–25.9
HPV16	25	9	5.5	2.2–13.5	13	5.8	2.7–12.5	12	63.9	16.4–248.1
Other cancer-associated types§	60	16	3.5	1.8–6.8	35	5.7	3.4–9.7	9	30.9	9.0–106.1
Low-risk types‖	72	25	4.0	2.3–6.9	25	3.2	1.8–5.3	8	6.8	2.1–22.3
HPV at diagnosis										
Negative‡	816	42	1	—	15	1	—	3	1	—
Positive‡	145	39	8.4	4.9–14.4	106	44.4	24.2–81.5	33	67.1	19.3–233.7
HPV16	30	12	14.6	6.1–34.8	21	48.4	20.6–113.5	16	371.8	61.7–2242.7
Other cancer-associated types§	43	18	18.5	8.6–39.7	63	90.5	44.1–185.9	11	273.9	42.2–1778.3
Low-risk types‖	72	9	3.8	1.7–8.6	22	20.0	9.4–42.6	6	33.2	6.1–181.6
HPV at enrollment/diagnosis										
Negative/negative	712	36	1	—	7	1	—	0		
Negative/positive	69	15	6.4	3.1–13.5	60	117.7	45.6–304.2	8	Not calculable¶	
Positive/negative	75	6	1.1	0.3–3.8	7	11.4	3.4–37.8	3	Not calculable¶	
Positive/positive	64	24	13.2	6.5–27.0	41	100.6	37.7–268.4	22	Not calculable¶	

*Adjusted for age (16–19, 20–29, 30–39, and ≥40 years), enrollment clinic (gynecology versus health appraisal), cytologic diagnosis at enrollment (benign reactive atypia or negative), participation status (returned enrollment questionnaire or not), follow-up time (<790 days versus ≥790 days), number of Pap smears during follow-up, and DNA testing laboratory (Cetus Corporation [Emeryville, CA] versus Albert Einstein College of Medicine [Bronx, NY]).

†ASCUS = atypical squamous cells of undetermined significance; SIL = squamous intraepithelial lesions.

‡Specimens with a very weak signal with the generic probe, and no type-specific signals, were excluded to minimize the possibility of misclassification of HPV status for these samples. Those whose polymerase chain reaction products hybridized clearly with the generic probe but none of the type-specific oligonucleotide probes were included as HPV positive, type undetermined.

§Including HPV types 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

‖HPV types other than HPV 16, 18, 31, 33, 35, 39, 45, 51 52, 56, 58, 59, and 68, including the undetermined types.

¶Relative risk estimates of infinity resulted because no cases of high-grade SIL were HPV negative at both enrollment and diagnosis.

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times. About the same proportion of control subjects turned from HPV positive to HPV negative, as vice versa. Only about 40% of those individuals who tested HPV positive at enrollment still remained positive at the follow-up visit. In contrast, among women diagnosed with new cervical neoplasia, the prevalence of HPV increased substantially at diagnosis compared with enrollment. In particular, approximately 90% of both low-grade SIL and high-grade SIL case patients tested positive for HPV DNA at diagnosis. Therefore, the ORs associated with HPV detection at diagnosis were much higher than at enrollment when all the women were still cytologically normal.

At diagnosis, analyses by HPV type groups showed that the risks were far higher among those detected with cancer-associated types than among those with only low-risk HPV types. Among the cancer-associated types, the higher risk associated with HPV16 compared with other cancer-associated types was evident at diagnosis only for high-grade SIL.

Results of repeated HPV measurement showed roughly similar patterns for ASCUS, low-grade SIL, and high-grade SIL (Table 1). Specifically, the highest risks were found among those who tested HPV positive at both times, followed by those with HPV DNA detected only at diagnosis, with less elevated risks observed among those with HPV DNA detected only at enrollment.

The effects of viral persistence appeared to vary somewhat by case subject group. Viral persistence, defined crudely as detection of the same HPV type at both times, was not associated with higher risks of ASCUS or low-grade SIL compared with HPV detection at both times but with different types (data not shown). For high-grade SIL, about two thirds of the women were HPV positive at both times. Among these high-grade SIL case patients who were HPV positive twice (compared with those control subjects who were positive twice), persistence of any cancer-associated HPV type was associated with a twofold risk compared with repeated detection of HPVs at both enrollment and diagnosis without a persistent cancer-associated type (data not shown). However, the numbers of high-grade case patients in each category of this analysis were less than 10.

There were 23 HPV types detectable by both the Cetus and Albert Einstein College of Medicine protocols. In Table 2, the relative frequencies of each of the 23 individual HPV types among those women who tested HPV positive at enrollment are presented. In the table, the women are presented according to their final case–control status, but the HPV results are derived from enrollment specimens to show the distribution of HPV types in advance of subsequent SIL.

Among those who remained cytologically normal throughout the study, HPV16 was the most common type (20.2% of HPV-positive control subjects), followed by HPV53 (16.2%). Conversely, among those who developed ASCUS, HPV53 was the most prevalent type (35.1%) at enrollment, followed by HPV16 (24.3%). Among women developing either low-grade SIL or high-grade SIL, HPV16 was detected most frequently. HPV51 was the second most common among patients who developed low-grade SIL. Other than HPV16, no type contributed to more than three patients who developed high-grade SIL.

In Table 3, the risks associated with HPV detection at enrollment are shown for women diagnosed subsequently with SIL at different lengths of time of follow-up. Among high-grade SIL case patients, the effect of follow-up time was important. No cases of new high-grade SIL developed in the first 766 days of follow-up among women who were HPV negative at enrollment (the earliest three occurred at days 767, 770, and 784 of follow-up). In other words, virtually all case patients with high-grade SIL within the first half of follow-up were HPV positive at enrollment, while only about half of the later-ascertained high-grade SIL case patients were HPV positive at enrollment. We found no convincing relationship between HPV detection at enrollment and age of the subject with the high-grade SIL, the size of the lesion estimated from the pathology notes, or the histologic grade (CIN 2 versus CIN 3) (data not shown).

Discussion

In this incident case–control study nested within a large cohort, we demonstrated that HPV infection, as measured by HPV DNA detection, greatly increases the risk of subsequent cervical SIL. The HPV test results predicted the cytologic future of the subjects, who were all initially normal. Our results support the temporal relationship between HPV infection

Table 2. Detection of selected individual virus types among human papillomavirus (HPV)-positive women at enrollment*

	Control subjects		Patients with ASCUS		Patients with low-grade SIL		Patients with high-grade SIL	
HPV type	No.	%†	No.	%†	No.	%†	No.	%†
HPV6/11	12	9.7	5	13.5	3	4.7	2	8.3
HPV16	25	20.2	9	24.3	13	20.3	12	50.0
HPV18	8	6.4	1	2.7	3	4.7	3	12.5
HPV26	1	0.8	1	2.7	0	0	0	0
HPV31	9	7.2	2	5.4	7	11.0	1	4.2
HPV33	6	4.8	1	2.7	3	4.7	1	4.2
HPV35	3	2.4	2	5.4	3	4.7	1	4.2
HPV39	7	5.6	2	5.4	5	7.8	2	8.3
HPV40	1	0.8	3	8.1	4	6.2	2	8.3
HPV45	9	7.2	1	2.7	2	3.1	0	0
HPV51	18	14.5	7	18.9	11	17.2	1	4.2
HPV52	8	6.4	3	8.1	3	4.7	2	8.3
HPV53	20	16.2	13	35.1	8	12.5	1	4.2
HPV54	5	4.0	5	13.5	3	4.7	1	4.2
HPV55	0	0	0	0	3	4.7	1	4.2
HPV56	5	4.0	4	10.8	8	12.5	2	8.3
HPV58	9	7.2	2	5.4	6	9.4	2	8.3
HPV59	7	5.6	0	0	0	0	3	12.5
HPV66	5	4.0	1	2.7	2	3.1	0	0
HPV68	2	1.6	3	8.1	3	4.7	0	0
PAP155	7	5.6	2	5.4	5	7.8	0	0
PAP238A	9	7.2	1	2.7	1	6.3	1	4.2

*ASCUS = atypical squamous cells of undetermined significance; SIL = squamous intraepithelial lesions.

†Percentage among HPV-positive subjects, excluding those who had only undetermined HPV types, since the analysis in this table is type specific. The denominators for these percentage calculations are as follows: control subjects (n = 125); patients with ASCUS (n = 37); patients with low-grade SIL (n = 64); patients with high-grade SIL (n = 24).

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Table 3. Odds ratios (ORs) and 95% confidence intervals (CIs) for cervical neoplasia associated with human papillomavirus (HPV) infection at study enrollment, by follow-up time*,†

Follow-up time, HPV −/+	No. of control subjects	Patients with ASCUS			Patients with low-grade SIL			Patients with high-grade SIL		
		No.	OR	95% CI	No.	OR	95% CI	No.	OR	95% CI
<510 days										
Negative	204	10	1		20	1		0		
Positive	58	13	8.3	2.7–25.3	32	8.4	4.0–17.5	6	Not calculable‡	
510–789 days										
Negative	201	22	1		26	1		3	1	
Positive	41	13	5.3	2.2–12.8	14	3.4	1.5–7.8	8	19.3	3.9–95.2
790–1119 days										
Negative	215	2	1		19	1		5	1	
Positive	32	13	3.8	1.6–9.1	13	4.4	1.8–10.9	6	8.7	1.8–42.9
≥1120 days										
Negative	214	46	1		35	1		5	1	
Positive	26	11	2.9	1.2–6.8	14	3.6	1.5–8.5	9	20.7	5.2–82.6

*Adjusted for age (16–19, 20–29, 30–39, and ≥40 years), enrollment clinic (gynecology versus health appraisal), cytologic diagnosis at enrollment (benign reactive atypia or negative), participation status (returned enrollment questionnaire or not), follow-up time (<790 days versus ≥790 days), number of Pap smears during follow-up, and DNA testing laboratory (Cetus Corporation [Emeryville, CA] versus Albert Einstein College of Medicine [Bronx, NY]).

†ASCUS = atypical squamous cells of undetermined significance: SIL = squamous intraepithelial lesions.

‡A relative risk estimate of infinity resulted because no case patients with high-grade SIL that was within 510 days were HPV negative at enrollment.

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and subsequent cervical neoplasia, further confirming that the relationship is a causal one. An elevated risk of future cervical neoplasia associated with the sensitive detection of HPV DNA in normal women is consistent with previous, smaller cohort studies [reviewed in *(3)*].

Compared with other cancer-associated HPV types, HPV16 infection predicted an especially elevated risk of high-grade SIL. Although HPV16 may account for a substantial portion of high-grade SIL (and cancer), many types of HPV seem nearly as likely to cause ASCUS and low-grade SIL.

The risks of SIL associated with HPV detection at enrollment did not show any convincing increases with age (with the possible exception of low-grade SIL, data not shown). We had predicted a stronger association among older women, because HPV DNA detection among cytologically normal women decreases strongly with age in many populations, including the Kaiser Permanente cohort studied here *(20,21)*. In contrast, women with SIL and cancer remain HPV DNA positive regardless of age *(22)*. Therefore, HPV DNA detection might be expected to be more highly associated with cervical SIL and cancer among women at older ages. Although we were limited to only six cases of high-grade SIL occurring in women more than 40 years of age, we found no such trend (only three of six were positive at enrollment). The lower than expected HPV detection cannot be explained by our data but may have been affected by our choice of collection instrument (lavage) and our unusual high-grade SIL case patients (rapid onset). The predictive value of cytology combined with HPV DNA testing among middle-aged and older women is being targeted in cohort studies now under way.

The magnitudes of risk estimates associated with HPV infection at diagnosis were much greater than at enrollment, because the HPV DNA prevalence among case patients, particularly among low-grade SIL case patients, increased substantially at diagnosis, while the prevalence among control subjects remained unchanged. The much higher prevalence of HPV infection at diagnosis suggests that many of the HPV infections among the case patients (especially those with low-grade SIL) were acquired during the follow-up after enrollment. In turn, the possibility of infection acquired during follow-up implies that the duration between initial HPV infection and detection of new SIL can often be even shorter than the approximately 2-year median time to diagnosis of our case patients.

Moreover, because the enrollment measurement of HPV infection could not pinpoint the time of infection (some of the infections might have already been persistent), we could not calculate the complete incubation curve from the time of infection to the diagnosis of SIL. The typically delayed diagnosis of SIL following its time of true development make incubation curves even more suspect. Nonetheless, it is clear that there is a large increase in risk of SIL in the first few years following HPV infection. An average incubation period of a few months to a few years would be analogous to the reported latency of genital warts following infection with HPV6 and HPV11 *(23)*.

The results of PCR measurement at both enrollment and diagnosis showed that the risks of SIL were highest among those who tested positive at both times. Those who had evidence of viral persistence with a cancer-associated HPV type, i.e., at least one cancer-associated HPV type that was detected at both times, were at an even higher risk of high-grade SIL diagnosis. Repeated detection of HPV16, the most common cancer-associated HPV, was associated with a particularly high risk of high-grade SIL, confirming other reports that a persistent or repeated infection with a cancer-associated type of HPV strongly increases the risk of high-grade SIL *(9,10)*.

In our analysis of two-time measurements, we were not able to differentiate persistent infection from recurrent infection with HPV of the same type. Future studies should incorporate HPV variant analysis methods to evaluate persistence, differentiate specific variants, and evaluate their importance separately *(24)*. As another concern, all high-grade SIL case patients in our study were incident case patients without previous detection of low-grade SIL or even ASCUS. The median number of Pap smears performed in the interval between enrollment and diagnosis was one (range, 0–5) for the high-grade SIL case patients as for all subjects combined. It is possible that the pathologic pathways of these rapid-onset cases of

high-grade SIL may be different from those that progressed via ASCUS or low-grade SIL over a longer period of time.

In conclusion, HPV DNA detection precedes and predicts the first cytologic detection of SIL. Many HPV types apparently cause low-grade SIL, whereas HPV16 predominates in the rarer rapid development of high-grade SIL without a preceding ASCUS or low-grade SIL. None of the behavioral risk factors for cervical neoplasia fundamentally altered the central role of HPV infection (data not shown). Possible clinical applications of HPV DNA testing and primary prevention of cervical cancer by vaccination are supported by firm etiologic evidence and should be defined by clinical studies.

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Notes

Present addresses: K.-L. Liaw, Department of Epidemiology, University of Pittsburgh, PA; M. M. Manos, Kaiser Permanente Division of Research, Oakland, CA; C. E. Greer, Chiron Corporation, Emeryville, CA; D. Tabor, Allied Technology Group, Rockville, MD.

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