Synergy between Cigarette Smoking and Human Papillomavirus Type 16 in Cervical Cancer *In situ* Development

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Abstract

Background: **A majority of studies have implicated the involvement of cigarette smoking in cervical cancer development, although its mechanism of action remains unclear. We conducted a large population-based case-control study to address the potential interaction between smoking and human papillomavirus type 16 (HPV-16) in development of cervical cancer *in situ* (CIS).**

Methods: **Information on risk factors for CIS was collected via interview, and archival cervical smears were tested for HPV-16 DNA presence in cases (n = 375) and controls (n = 363). Adjusted odds ratios (OR) for the effects of smoking, HPV-16 presence/absence, and load at first smear (taken, on average, 9 years before diagnosis) were calculated.**

Results: **The risk for CIS among current smokers who were HPV-16 positive at time of first smear was >14-fold [adjusted OR, 14.4; confidence interval (95% CI), 5.6-36.8] compared with HPV-16-negative current smokers. In contrast, the risk for CIS among HPV-16-positive nonsmokers was only 6-fold (adjusted OR, 5.6; 95% CI, 2.7-11.5), compared with HPV-16-negative nonsmokers. HPV-16-positive smokers with high viral load at time of first smear exhibited a high risk for CIS (adjusted OR, 27.0; 95% CI, 6.5-114.2) compared with HPV-16-negative smokers. Within nonsmokers, however, high HPV-16 load contributed only a 6-fold increased risk compared with HPV-16-negative nonsmokers (adjusted OR, 5.9; 95% CI, 2.4-14.6). Interaction was observed (P = 0.03) between duration of smoking and HPV-16 presence in CIS development.**

Conclusion: **Results suggest a synergistic effect between smoking and both HPV-16 status and HPV-16 viral load, which may occur almost a decade before CIS detection.** (Cancer Epidemiol Biomarkers Prev 2006;15(11):2141–7)

Introduction

Cervical cancer remains one of the leading causes of cancer mortality globally, predominantly in less developed countries (1). It is widely accepted that certain ''oncogenic'' types of human papillomavirus (HPV) are necessary causes of cervical cancer development. Of the 13 to 19 proposed oncogenic HPV types, HPV-16 exhibits the highest prevalence (46-63%) in cervical cancers in the majority of studies (2, 3).

Presence of HPV infection cannot be considered a sufficient causative agent due to the numbers of HPV-infected women who do not develop cervical cancer. The roles of other potential risk factors in cervical carcinogenesis therefore need to be considered. An increased risk of cervical cancer associated with tobacco smoking has been established on the basis of a number of epidemiologic studies since the 1980s (4, 5). Whether this link is related to genotoxic DNA adducts of smoking in the cervix epithelium (6-8), its effect on malignant transformation of HPV-infected cells (9), or its influence on HPV infections via localized immunosuppression (10) has been discussed. It has also been debated whether an association of smoking and cervical cancer is merely an artifact of confounding by HPV because of association of smoking with sexual activity and the subsequent risk of acquiring an HPV infection (11, 12). A number of studies, restricted to HPV-positive women, have shown an increased risk for cervical cancer in smokers compared with nonsmokers (13-19), but to our knowledge, only three studies have formally tested for interaction between HPV and smoking in cervical cancer development (15, 20, 21), two of which seem to have small numbers (20) or a less appropriate study design for detecting interaction (20, 21). Furthermore, to our knowledge, there seems to be a lack of information about the combined effects of HPV load and smoking in cervical cancer development.

A previous study in our group by Ylitalo et al. (22) investigated the possible roles of various smoking attributes stratified by presence of HPV-16/HPV-18. The risk of cancer *in situ* (CIS) associated with various smoking attributes was more pronounced in HPV-positive women than in HPV-negative women. We were interested in statistically testing whether our previous finding was, in fact, the result of an interaction between HPV and smoking.

Moreover, subsequent to the study by Ylitalo et al. (22), further information was acquired on HPV-16 viral load for those women exhibiting HPV-16 infections in any archival smears collected over a 25-year period. Using this additional information in our current study, we were able to study both the individual and synergistic effects of HPV-16 load and tobacco smoking many years before diagnosis of CIS when the cervical smears were taken. We also used a proxy for persistence to examine how a multiple number of HPV-16-infected smears related to a woman's risk of cervical cancer *in situ* (CIS).

Materials and Methods

Subjects. The source population for this population-based case-control study was composed of all Swedish women (n = 146,104) who participated in cytologic screening in Uppsala

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county at any time during the years 1969 to 1995. All smears were stored at the Uppsala University Hospital and, given the publicly funded nature of Swedish health care, equal access for women to health facilities could be assumed.

Within this source population, a cohort of 105,760 women was identified who possessed the following characteristics: (*a*) their first registered smear during the study period was cytologically normal (PAP = 1); (*b*) they were born in Sweden; and (*c*) they were <50 years of age at entry to the cohort (date of the first smear). Using the Swedish cancer registry, all CIS cases within the cohort were identified. For each CIS case, one control, individually matched on date of entry into cohort (± 3 months) and age, was identified from the cohort. These controls were free of malignant disease of the cervix and had not undergone a hysterectomy before the time (and age) of CIS diagnosis for their matched case. Four hundred ninety-nine eligible cases and their 499 matched controls remained after review of the cases histologic slides by an experienced pathologist to confirm the CIS diagnosis. Each case woman and her matched control were considered a risk set.

Ethical approval was obtained from the Medical Faculty Research Ethics Committee at Uppsala University and from the Karolinska Institutet Review Board.

HPV Analyses. All samples from cases and controls within risk sets were recoded and mixed to ensure blinding during analysis. DNA extraction from cervical slides was done using a previously described protocol (23). PCR-based detection of HPV-16 and quantification of HPV-16 load were done on DNA from all cervical slides for each woman using real-time detection of accumulated fluorescence (TaqMan) as previously described (24). Those women with only β-actin-negative smears during follow-up (12 cases and 20 controls) were excluded from our analyses. Of the 3,771 smears tested, 2,970 (78.8%) tested positive for β-actin. To omit smears taken as part of the diagnostic workup, we also disregarded all smears taken within 1 year before the CIS diagnosis date for cases or their matched controls in the subsequent statistical analyses. Consequently, 35 case and 32 control women who possessed only a single smear taken within 1 year before diagnosis were removed from analyses.

HPV-16 infection was considered present for a woman whose β-actin-positive smear was also positive for HPV-16 DNA. HPV-16 load per smear was calculated using the ratio of cycle threshold (CT) values for HPV-16 DNA to β-actin DNA created in the real-time PCR. Simply put, a decreasing CT ratio corresponds to an increasing level of HPV-16 DNA in a given sample. A median value was determined from CT ratios of HPV-16-infected control women and categories of ''low'' and ''high'' HPV-16 load were defined as being values above or below these median values, respectively.

Interview Data. Detailed information about smoking habits (age at starting, duration, intensity) and other covariates (oral contraceptive use, parity, age at sexual debut, number of sexual partners, socioeconomic factors) was collected from cases and controls via telephone interviews. The interviewers were blinded on case-control status. The methods for collection of this data have been previously described for this study (22). Thirty cases and their matched controls from the eligible 499 risk sets were excluded because they did not have access to a telephone. Of the 469 cases and 469 controls approached by telephone, 422 (90%) case women and 422 (90%) control women agreed to participate.

Statistical Analyses. To improve the power to detect interaction between smoking and HPV-16 in CIS development (as well as main effects), we used unconditional logistic regression in our analyses to produce odds ratios (OR). We interpreted these ORs as estimates of relative risk. Because matching was done on several variables, we treated the data as group matched on age and number of years between first eligible (β-actin positive and collected >1 year before diagnosis of CIS) smear and diagnosis. Comparisons were made with analyses done using conditional logistic regression, taking into account the matching criteria. As expected, these risk estimates did not differ appreciably to those generated using unconditional logistic regression. We therefore report results from unconditional analyses because this enabled us to use incomplete risk sets in our analyses to gain precision.

We chose to use the first eligible smear for our analyses to study the joint effect of HPV-16 and smoking at an early stage during carcinogenesis.

In our analyses, a ''current smoker'' was defined as someone who smoked within 2 years before the date that a given smear was taken. A 2-year period was chosen to try and capture relatively short-term effects of smoking. The corresponding ''nonsmoking'' group was a combination of women who either had never smoked or who had not smoked within 2 years before a given smear being taken. ''Duration of smoking'' was categorized based on years of smoking before first smear (0, <5, and ≥5 years). Smoking intensity was measured as pack-years at time of first smear (0, <2.5, and ≥2.5 pack-years).

Covariates included in the multivariable model were age, number of years between first smear and diagnosis, current oral contraceptive use (never or >2 years before smear, within 2 years before smear), parity (nulliparous, 1, 2, and 3), and number of sexual partners up to the date of the first smear. Other potential confounders were not included as covariates in the model as they were considered surrogate measures for the number of sexual partners (marital status and sexual debut) and/or inclusion/exclusion from the multivariable analysis did not appreciably alter the risk estimates for the main exposure variable in the model.

We derived a variable to be used as a surrogate marker for persistence of HPV-16 infection based on the proportion of total smears collected for each woman that were HPV-16 positive, also taking into account the number of smears per woman. The number of HPV-16-positive smears per woman was divided by the square root of the total number of smears per woman. HPV-16 proportion = number of HPV-16-positive smears per woman / $\sqrt{}$(total number of smears per woman). E.g., 2 HPV-16-positive smears of a total of 2 smears will be ranked lower than a woman with 5 HPV-16-positive smears of a total of 5. Their respective values in this example would be 1.4 and 2.2. A woman with 2 HPV-16-positive smears of a total of 5 smears would have a lower value than the previous examples (0.9).

We used the square root of the total number of smears in the denominator to account for the differences in number of smears per woman. Clearly, a woman with 5 smears that are all positive for HPV-16 is more likely to have a truly persistent infection than an individual possessing only 2 smears, both of which were positive. Likewise, it seems more plausible that a woman with 2 of 2 smears (HPV-16-positive smears / total number of smears) possesses a persistent infection than a woman possessing 2 of 5 smears that are HPV-16 positive.

Women were considered to have a low proportion (persistence marker) of HPV-16 infections if their calculated value was <0.5, where 0.5 was the median value observed in HPV-16-positive nonsmokers. Women possessing an HPV-16 proportion (persistence marker) value ≥0.5 were considered to have a high proportion of HPV-16 infections.

Tests for interaction were based on departure from multiplicativity of the ORs associated with smoking and HPV-16. A multiplicative logistic regression model was used, where the OR for HPV-16 or HPV-16 load was allowed to vary for different levels of smoking status, duration, or intensity. This meant that an interaction effect was added to the main effect.

Table 1. Characteristics of 738 participants used in first smear analyses

Characteristic	CIS cases (N = 375), n (%)	Controls (N = 363), n (%)	P*
Matching variables			
Age at first smear (y)			
≤20	38 (10.1)	35 (9.6)	—
20-25	116 (30.9)	113 (31.1)	
26-30	113 (30.1)	111 (30.6)	
>30	108 (28.8)	104 (28.6)	
No. years between first smear and diagnosis			
<5	130 (34.7)	123 (33.9)	—
5-9	67 (17.9)	76 (20.9)	
10-14	87 (23.2)	77 (21.2)	
≥15	91 (24.3)	87 (24.0)	
Other risk factors			
Marital status at diagnosis			
Married	278 (74.1)	281 (77.4)	0.71
Unmarried	43 (11.5)	41 (11.3)	
Divorced	39 (10.4)	31 (8.5)	
Widowed	15 (4.0)	10 (2.8)	
Education (y) at diagnosis			
6-9	114 (30.4)	88 (24.2)	0.18
10-12	102 (27.2)	107 (29.5)	
≥13	159 (42.4)	164 (45.2)	
Missing	0	1 (1.1)	
Oral contraceptive use at first smear			
Never/prior	153 (40.8)	180 (49.6)	0.01
Current†	222 (59.2)	183 (50.4)	
Parity at first smear			
Nulliparous	141 (37.6)	180 (49.6)	<0.001
1	101 (26.9)	71 (19.6)	
2	100 (26.7)	73 (20.1)	
≥3	33 (8.8)	39 (10.7)	
Age at sexual debut (y)			
≤15	92 (24.5)	92 (25.3)	0.55
16-17	154 (41.1)	136 (37.5)	
18-19	91 (24.3)	84 (23.1)	
≥20	38 (10.1)	51 (14.0)	
No. sexual partners at first smear			
0-1	66 (17.6)	103 (28.4)	<0.001
2-3	130 (34.7)	125 (34.4)	
4-5	85 (22.7)	75 (20.7)	
≥5	94 (25.1)	60 (16.5)	

*P values adjusted for age at first smear and number of years between first smear and diagnosis.
†Current, within 2 years before the smear date.

Tests for trend were done using the Wald test. This was used to test whether a linear trend existed with respect to the log odds for a given exposure. These and the aforementioned analyses were done using SAS version 9.1.3.

Results

Of the 844 women (422 cases and 422 controls) from whom we had interview data, 738 (375 cases and 363 controls) were included in all statistical analyses apart from the analyses using our surrogate persistence marker. These women had an HPV-16 result in at least one β-actin-positive smear collected >1 year before diagnosis of a CIS lesion. Women were included in the persistence surrogate analysis (256 cases and 224 controls) if they had two or more smears in addition to having satisfied the aforementioned criteria. For both cases and controls, the median number of smears per woman was 3 (range, 2-16 in cases and 2-14 in controls). The mean numbers of smears per woman were 3.98 and 3.57 for cases and controls, respectively.

Characteristics of the 738 participants are presented in Table 1. The median age at first smear was 25.5 years (range, 15-49 years) and the median time between first smear and diagnosis was almost 9 years (range, 1-25 years). The risk of CIS increased in relation to current oral contraceptive use (P = 0.01), parity of 1 to 2 children (P < 0.001), and increasing number of sexual partners (P < 0.001).

Multivariable analyses (Table 2) showed that women positive for HPV-16 in their first smear had an 8-fold increased risk of CIS [adjusted OR, 8.4; confidence interval (95% CI), 4.8-14.7] compared with women negative for HPV-16. A strong association and trend was observed for HPV-16 load and risk of CIS. Having low or high HPV-16 load in the first smear corresponded to an increased adjusted risk of 5.5 (95% CI, 2.4-12.6) or 11.0 (95% CI, 5.3-22.6), respectively (P_{trend} < 0.0001) compared with being HPV-16 negative. Likewise, having a high proportion of HPV-16-positive smears per total number of smears increased a woman's relative risk for CIS when compared with HPV-16-negative women (adjusted OR, 11.8; 95% CI, 7.2-19.5). Those with a low proportion of HPV-16-positive smears showed no increased risk (adjusted OR, 0.8; 95% CI, 0.3-2.0) compared with HPV-16-negative women.

Current smoking at time of first smear conferred a 70% increase in risk for development of CIS (adjusted OR, 1.7; 95% CI, 1.2-2.4) compared with nonsmokers (Table 2). The adjusted ORs for smoking debut, intensity, and duration were similar to those seen for current smoking.

In Table 3, the joint effect of HPV-16 and smoking at time of first smear is shown. When stratified by current smoking status, nonsmoking women who were positive for HPV-16 had a 5-fold increased risk (adjusted OR, 5.6; 95% CI, 2.7-11.5) of CIS compared with nonsmoking HPV-16-negative women. However, being a smoker and HPV-16 positive was related to a 14-fold increased risk (adjusted OR, 14.4; 95% CI, 5.6-36.8) compared with HPV-16-negative nonsmokers. Nonsmoking

Table 2. OR and 95% CI of CIS in relation to HPV-16 and smoking habits

Variable	Cases/controls	Crude OR (95% CI)	Adjusted OR* (95% CI)
HPV-16 status			
Negative	268/346	Reference	Reference
Positive	107/17	8.4 (4.9-14.5)	8.4 (4.8-14.7)
HPV-16 load			
Negative	268/346	Reference	Reference
Low	31/8	5.1 (2.3-11.3)	5.5 (2.4-12.6)
High	76/9	11.4 (5.6-23.4)	11.0 (5.3-22.6)
P_{trend} (Wald)			P < 0.0001
HPV-16 proportion/persistence†			
Negative	94/181	Reference	Reference
Low	8/18	0.8 (0.3-2.0)	0.8 (0.3-2.0)
High	154/25	12.2 (7.4-19.9)	11.8 (7.2-19.5)
Smoking status‡			
Nonsmokers	131/181	Reference	Reference
Current smokers	244/182	1.9 (1.4-2.6)	1.7 (1.2-2.4)
Age at smoking debut (y)			
Never	110/162	Reference	Reference
≥16	162/128	1.9 (1.3-2.6)	1.8 (1.2-2.5)
<16	103/73	2.2 (1.4-3.2)	1.7 (1.1-2.6)
Smoking duration (y)			
0	119/171	Reference	Reference
<5	61/56	1.5 (1.0-2.4)	1.5 (0.9-2.4)
≥5	195/136	2.1 (1.5-2.9)	1.7 (1.2-2.4)
Smoking intensity (pack-years)			
0	119/171	Reference	Reference
<2.5	116/88	1.9 (1.3-2.8)	1.7 (1.2-2.6)
≥2.5	140/104	1.9 (1.4-2.7)	1.6 (1.1-2.3)

*ORs are adjusted for age at first smear, number of years between date of smear and diagnosis, current oral contraceptive use, and parity as categorized in Table 1. Smoking-associated variables were adjusted for HPV-16 status (negative, positive) and HPV-16-associated variables were adjusted for current smoking status (nonsmoker, current). Number of sex partners before first smear was included as a continuous variable.
†Categories for HPV-16 proportion: 0, <0.5, ≥0.5.
‡Current smokers, subject smoked within 2 years before the smear date; nonsmokers, subject never smoked or smoked >2 years before the smear date.

Table 3. Multivariable analysis of CIS in relation to HPV-16 status at first smear, stratified by smoking status

Variable	Nonsmokers*		Current smokers†		$P_{interaction}$
	Cases/controls	OR‡ (95% CI)	Cases/controls	OR‡ (95% CI)	
HPV-16 status					
Negative	96/169	Reference	172/177	Reference	0.12
Positive	35/12	5.6 (2.7-11.5)	72/5	14.4 (5.6-36.8)	
HPV-16 load					
Negative	96/169	Reference	172/177	Reference	0.21
Low	12/5	5.1 (1.7-15.4)	19/3	6.0 (1.7-20.8)	
High	23/7	5.9 (2.4-14.6)	53/2	27.0 (6.5-114.2)	
P_{trend}		$P < 0.0001$		$P < 0.0001$	

*Nonsmokers, subject never smoked or smoked >2 years before the smear date.
† Current smokers, subject smoked within 2 years before the smear date.
‡ ORs adjusted for age at first smear, number of years between date of smear and diagnosis, current oral contraceptive use, and parity as categorized in Table 1. Number of sex partners before first smear was included as a continuous variable.

women with a high viral load had an ~6-fold increased risk (adjusted OR, 5.9; 95% CI, 2.4-14.6) compared with nonsmoking HPV-16-negative women. In comparison, current smokers with a high HPV-16 viral load had an increased risk of 27.0 (95% CI, 6.5-114.2) compared with current smokers without HPV-16 infection. Tests for interaction on a multiplicative scale between HPV-16 infection and current smoking status ($P = 0.12$), or HPV-16 load and current smoking status ($P = 0.21$), were not significant.

The potential interaction between HPV-16 and smoking was further explored using other indicators of smoking status (duration and intensity; Table 4). In stratified analyses, the strongest risk was observed among high-duration smokers (≥5 years) who were positive for HPV-16 infection at time of first smear, compared with HPV-16-negative, high-duration smokers (adjusted OR, 35.9; 95% CI, 8.6-150.2). The corresponding risk among nonsmokers who were HPV-16 positive was 4.8 (95% CI, 2.2-10.3). Presence of a multiplicative interaction between duration of smoking and HPV-16 status was found ($P = 0.03$; Table 4). Smoking intensity followed a similar risk pattern but did not show significance in a formal test for interaction (Table 4).

Discussion

We present data which suggest an early synergistic effect between smoking and HPV-16 in CIS development. Our results show that the risk for CIS among HPV-16-positive current smokers at time of first smear (on average, 9 years before diagnosis of CIS) was >14-fold, compared with women who were HPV-16-negative current smokers at time of first available smear. Current smokers with a high HPV-16 viral load at time of first smear were at a particularly increased risk (27-fold) compared with current smokers without HPV-infection. Within nonsmokers, however, high HPV-16 load contributed only a 6-fold increased risk compared with HPV-16-negative nonsmokers at time of first smear. Interaction on a multiplicative scale was observed ($P = 0.03$) between duration of smoking and HPV-16 presence at time of first smear in CIS development.

Initial analyses centered on confirming that current smoking was an independent risk factor for cervical cancer. In our study, we used women's current smoking status at the time a smear was taken, rather than at CIS diagnosis or end of follow-up, which many case-control studies use. This is of particular importance when considering that one of our main hypotheses related to the potential interaction between smoking and HPV-16 in causing CIS. Clearly, both exposures need to be present at the same time for there to be an interaction. Evidence for interaction would imply a synergistic action between HPV and smoking that would greatly increase the likelihood of women developing CIS if they are HPV-positive smokers and would put them in a risk group worthy of careful monitoring.

Our adjusted risk estimates for current smoking in association with CIS are in agreement with a majority of studies examining the effect of smoking on risk for developing cervical cancer (14, 25). Given the adjustment for most known CIS risk factors in our analyses, the association between smoking and CIS is unlikely to be explained by confounding.

To examine whether the risk of CIS could be explained by an interaction between HPV-16 and smoking, we did analyses stratified by current smoking at time of first smear (Table 3). Clearly, the risk of CIS in relation to HPV-16 status differed between nonsmoking and smoking women. This difference was even more pronounced for HPV-16 load risk estimates in different smoking categories. Based on these results, it is tempting to suggest that the increased risk observed in the smoking group is mediated through its influence on HPV-16 viral load. High HPV-16 viral load in this and other studies has been shown to be associated with increased risk for CIS (24, 26-29). Alternatively, perhaps a certain load of HPV-16 is necessary for smoking to have an effect.

Subsequently, the effects of HPV-16 on CIS within categories of number of pack-years and smoking duration up to first smear were examined (Table 4). Again, the risk of CIS in relation to HPV-16 status differed between smoking strata, suggesting an interactive effect between smoking and HPV-16. Although based on small numbers, we found a statistically

Table 4. Multivariable analysis of CIS in relation to HPV-16 status in first smear, stratified by smoking attributes at first smear

Variable	HPV-16 status	No. cases/controls	Adjusted OR* (95% CI)	$P_{interaction}$
Smoking duration (y)				
0	Negative	91/160	Reference	0.03
	Positive	28/11	4.8 (2.2-10.3)	
<5	Negative	49/52	Reference	
	Positive	12/4	3.3 (1.0-11.2)	
≥5	Negative	128/134	Reference	
	Positive	67/2	35.9 (8.6-150.2)	
Smoking intensity (pack-years)				
0	Negative	91/160	Reference	0.12
	Positive	28/11	4.8 (2.2-10.2)	
<2.5	Negative	86/84	Reference	
	Positive	30/4	7.9 (2.6-23.7)	
≥2.5	Negative	91/102	Reference	
	Positive	49/2	26.9 (6.3-114.6)	

*ORs are adjusted for age at first smear, number of years between date of smear and diagnosis, current oral contraceptive use, and parity as categorized in Table 1. Number of sex partners before first smear was included as a continuous variable.

significant multiplicative interaction between duration of smoking and HPV-16 presence causing CIS. Biological explanation for this possible interaction could involve the influence of smoking on persistence of infection or HPV-16 load. Persistence of carcinogenic HPV infections is a major topic of interest for many researchers in this field. Numerous studies have shown persistence of certain oncogenic HPV types to be a greater risk factor for cervical cancer than HPV presence alone (26, 30-33) and that different HPV types have varying propensities for persistence (34, 35). The effect of smoking on persistence of oncogenic HPV infections has been difficult to discern due to contradictory findings (15, 36-42). Most likely, this is due to differences in definitions for persistence, HPV type, study design, and sample sizes. In our study, we were unable to directly address the effect of smoking on persistence because the relationship between persistence and CIS would bias risk estimates if CIS cases were included in the analysis. Unfortunately, the low numbers of non-CIS (control) women possessing persistent infections did not allow us to address the issue using a less biased sample. Another limitation imposed on us by the relatively low numbers of persistent HPV-16-infected women was the inability to effectively investigate the interaction between HPV-16 persistence (using our surrogate marker for persistence) and smoking.

In our study population, archival smears were variable both in the number of total smears and the time interval between smears for each woman. Generally, researchers have defined persistence as the presence of HPV in two or more consecutive smears (preferably of the same type or allelic variant) over a given period of time (43). Given the sporadic nature of smears in our study, we felt the common definition for persistence was inappropriate. Rather, we took into account the proportion of HPV-16-infected smears per woman during the entire follow-up period. This allowed us to use HPV-16 information on all smears per woman to gain a summary of HPV-16 infection during the study.

Using this marker for persistence, we observed an increased risk for CIS associated with a high proportion of HPV-16-infected smears out of a total number of smears per woman. This apparent threshold effect, which occurred where proportions were greater than the median of controls, highlights the importance of persistent infections. Whether this or another persistence measure is more clinically relevant to use remains to be seen. We observed similar risk estimates for high HPV-16 load and HPV-16 proportion (our surrogate persistence marker). Considering that our HPV-16 load measurements are based on single smears taken many years before the end of follow-up, HPV-16 load may be more appropriate in a clinical setting for diagnosing women with a higher predisposition for CIS.

The role of smoking remains particularly enigmatic. If there is indeed an association between cigarette smoking and HPV incidence (and/or persistence), the mechanism may be related to localized immune suppression (44-46). Others have proposed means more related to neoplastic progression than to immunoregulation of HPV (13, 15). It is entirely possible that as a result of the strategies of HPV for evading the immune system, both of these mechanisms are of importance.

Studies have found that, as part of the ability of HPV to evade immune recognition, it can inhibit various components of the innate immune system (47-51), which may in turn promote a Th2-biased immune response. Such a shift in immunity would favor viral persistence rather than viral clearance and may aid tumor progression by subverting immune surveillance mechanisms (52). Evidence supporting this concept is the increased plasma concentrations of interleukin-10 and/or decreased levels of IFN-γ observed in HPV-positive women with cervical intraepithelial neoplasia (52). Interestingly, these cytokines have also been described in relation to their similar modulation by cigarette smoke substituents, along with a noteworthy inhibition of tumor necrosis factor-α in some studies (53, 54). Furthermore, association studies of the tumor necrosis factor-α promoter region have implicated the involvement of tumor necrosis factor-α in cervical intraepithelial neoplasia development (55, 56). This has been corroborated by the observation that tumor necrosis factor-α and IFN-γ act synergistically to destroy ME-180 cervical cancer cells via apoptosis or necrosis (57).

It would be premature to hypothesize that cigarette smoking exacerbates the carcinogenic potential of HPV specifically via stimulation of interleukin-10 or inhibition of IFN-γ (and/or tumor necrosis factor-α). However, the fact that some cigarette substituents have the ability to manipulate cytokine expression in a similar manner to HPV would allow the possibility that smoking may enhance the ability of HPV to avoid the immune system and, at the same time, increase the chances of neoplastic progression via further imbalance of the Th1 and Th2 cytokine profile. This might explain the contradictory nature of evidence supporting the role of smoking both in increasing incidence and persistence of HPV and in neoplastic progression.

The ability of HPV to manipulate cytokine levels, along with expression of the HPV E6 and E7 proteins, which are thought to exacerbate the oncogenic process, is likely to implicate HPV load as much as persistence of HPV. Some studies have shown a correlation between increasing HPV load and increasing numbers of E6 and E7 transcripts (17, 58). Transcript levels of these and other HPV proteins are likely to be important in development of cervical cancer.

Several issues need to be addressed on study design and limitations. Despite our study containing large numbers of women, relative to other studies on interaction, there were low numbers of HPV-16-positive female controls who also smoked. This meant that some strata contained relatively small numbers of individuals. A larger study, designed with a particular emphasis on recruiting more controls than were included in our study, would improve the power to detect significant interactions between smoking and HPV-16.

We found few articles that formally tested for either additive or multiplicative interaction between HPV and smoking in CIS formation (15, 20, 21). Differences in design and analysis in these studies make comparison difficult. Ideally, to examine interaction, it would be preferable to have concurrent information on active HPV infections and current smoking habits. The use of antibodies to HPV, as in the study by Hakama et al. (21), would not allow an accurate determination of HPV infection at time of sample retrieval because they may represent either a past or current infection. Similarly, the use of an ''ever'' or ''past'' classification for smoking is probably inadequate for testing interaction (20). Other notable differences in study design were that Olsen et al. (20) tested for interaction in a much smaller sample size than ours and Hakama et al. (21) used cervical cancer as an outcome, whereas our outcome of interest was CIS.

A study by Harris et al. (15) possessed a design more appropriate for testing interaction. They confirmed results from previous studies which showed a greater increased risk for smokers who were HPV infected compared with smokers who were not HPV infected. Furthermore, their analyses suggest a dose response between smoking and cervical cancer in women with persistent HPV infections. They were not, however, able to see evidence for an interaction between ''repeated'' oncogenic HPV positivity and amount of smoking in causing cervical intraepithelial neoplasia. It was not possible to do a comparable test for interaction between persistent HPV and smoking in our study due to lack of power. However, when we examined the associations of HPV-16 ''positivity'' and smoking duration with CIS development, there seemed to be a multiplicative interaction between HPV-16 presence and high duration (≥5 years) of smoking. Similar analyses of

smoking intensity (pack-years) were also suggestive of an interaction.

Another limitation of our study relates to a lack of information on infection with other HPV types than HPV-16 in the smears. This potentially prevents us from adjusting completely for HPV in the analyses of smoking as a main effect. However, we do adjust for number of sexual partners, which could be regarded as a good proxy for acquiring an HPV infection. Indeed, after including number of sexual partners to our model, adding HPV-16 only changes the adjusted risk estimates for smoking marginally. Because HPV-16 is the most prevalent HPV type and number of sexual partners is adequately adjusted for it in the model for smoking as a main effect, we should expect little remaining residual confounding by other HPV types. Of course, the possibility of recall bias must be mentioned because this was a case-control study with data for smoking and other risk factors (apart from HPV-16) being collected via telephone interview. Although we cannot discount the possibility of differential bias, perhaps it is lessened by the fact that interviews were often done well after diagnosis and treatment of the offending lesion. This might promote a more realistic recollection of history related to the various risk factors. There will, however, be some amount of nondifferential misclassification bias because we examined details of the women's histories, which are prone to misrecollection. This could bias the risk estimates towards the null and contribute to slightly lower estimates for smoking risk. We believe our method for detection of HPV-16 to be highly sensitive and specific. Therefore, misclassification of our exposure variable (HPV-16) should be minimal. Equally, we have minimized the possibilities of outcome misclassification by having one experienced pathologist examine all histologies in a blinded manner.

To conclude, our study revealed evidence suggestive of synergism between cigarette smoking (particularly duration of smoking) and HPV-16 in CIS development, which occurs many years before diagnosis of the offending lesion. This supports the results of a small number of studies that have formally tested for an interaction between smoking and HPV in causing CIS. Confirmation of an interaction between cigarette smoking and HPV-16 (and other HPV types) in cervical cancer development from other large-scale studies is of vital importance from a public health perspective, considering the widespread exposure to these agents in young women at risk for cervical cancer.

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