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HPV 16 AND CIGARETTE SMOKING AS RISK FACTORS FOR HIGH-GRADE CERVICAL INTRA-EPITHELIAL NEOPLASIA

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Although genital human papillomavirus (HPV) infection is well established as the etiologic agent for cervical intra-epithelial neoplasia (CIN), little is known about the cofactors involved in the development of high-grade lesions or the progression of low-grade to high-grade lesions. In our study of HPV-infected women with CIN (163 CIN I, 51 CIN II and 44 CIN III), women with CIN II or III were compared with those with CIN I for risk factors associated with high-grade lesions. After controlling for age, education, ethnicity and frequency of Pap smear screening, infection with HPV 16, but not high viral load or infection with multiple types, was associated with high-grade lesions (OR for CIN II = 11.96, OR for CIN III = 23.74). Risk of CIN III, but not CIN II, increased with number of cigarettes smoked per day (ORs = 1.49 and 3.35 for ≤10 and >10 cigarettes per day, respectively) and decreased with frequency of condom use during sex (ORs = 0.60 and 0.32 for women who used condoms occasionally/sometimes and most/all of the time, respectively). There were no associations between high-grade lesions and plasma levels of micronutrients (retinol, β-carotene, α-tocopherol and reduced ascorbic acid). Our results indicate that infection with HPV 16 is associated with high-grade lesions. Additional cofactors, such as cigarette smoking, may be required as a carcinogen to advance HPV-infected cells toward neoplastic progression. ***Int. J. Cancer*** **78:281–285, 1998.**
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Cervical intra-epithelial neoplasia (CIN) is histopathologically classified into grades I, II or III according to the extent of abnormality in the squamous epithelium. Many low-grade lesions (CIN I) spontaneously regress, yet some have the potential to progress to high-grade lesions (CIN II and III) and further to invasive cancer (Romney *et al.,* 1997; Syrjänen *et al.,* 1992). Some high-grade lesions, however, may develop without originating from low-grade lesions (Koutsky *et al.,* 1992). Genital human papillomavirus (HPV) infection is the major causal factor of cervical neoplasia (Koutsky *et al.,* 1992; Schiffman *et al.,* 1993), but little is known about the cofactors in addition to HPV that are involved in the progression of low-grade to high-grade lesions or that predispose to the development of high-grade lesions. Our study compared HPV-infected women with CIN I to those with CIN II and III to identify factors that differentiated women with low-grade lesions from those with high-grade lesions. Risk factors examined for association with high-grade lesions included characteristics of HPV infection (HPV type, number of HPV types and viral load), plasma levels of micronutrients and various life-style variables.

MATERIAL AND METHODS

CIN population

All women with an abnormal Pap smear diagnosed in primary health-care clinics affiliated with the Albert Einstein College of Medicine were referred for colposcopy and cervical biopsy. Women were recruited from colposcopy clinics in 1992–1994 to study the etiology of CIN. Eligibility criteria included having had a cervical biopsy and/or endocervical curettage on the day of recruitment for evaluation of an abnormal Pap smear, not being pregnant, no history of cancer and having an intact cervix. Of the 551 women recruited, 466 had CIN diagnosed by various staff pathologists at various clinic sites. Biopsy slides from these 466 cases were retrieved and reviewed by the study pathologist (A.S.K.), and CIN was histo-pathologically confirmed and graded in 378 cases. Since genital HPV infection is the major etiologic agent for CIN and is known to influence the natural history of CIN, our study focused on the identification of cofactors associated with high-grade CIN in 258 HPV-infected women among the 348 who had known HPV results, including 163 (67.9%) of 240 CIN I, 51 (87.9%) of 58 CIN II and 44 (88.0%) of 50 CIN III (including one case of cervical cancer).

Data collection

The protocol was approved by the institutional review board, and all study subjects gave informed consent. A questionnaire was administered by a trained interviewer, who was fluent in English and Spanish. Plasma was prepared from peripheral venous blood samples, which were wrapped in aluminum foil upon collection. Plasma levels of retinol, β-carotene, reduced ascorbic acid (active form) and α-tocopherol were measured by high-pressure liquid chromatography (HPLC), as described previously (Begrens and Madere, 1987; Palan *et al.,* 1991). The coefficient of variation for these assays was $< 8\%$.

Cervico-vaginal lavage samples were collected for HPV DNA testing by both PCR and Southern blot hybridization techniques, as described by Burk *et al.* (1996). A sample was considered HPV-positive if either PCR or Southern blot was positive. PCR samples that did not hybridize to any of the 39 type-specific probes were considered to have an ''uncharacterized'' type. An infection with a low viral load was defined as HPV DNA detectable by PCR only, whereas high-level infection was detectable by both Southern blot and PCR. HPV types determined by PCR and Southern blot were combined for analyses. To identify specific HPV types associated with severity of CIN, subjects were classified into 1 of 3 risk categories according to HPV type(s): *(i)* high risk, HPV 16; *(ii)* medium risk, HPV 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and W13b; and *(iii)* low risk, all other HPV types, including those uncharacterized. This classification of HPV was based on type-specific HPV prevalence found among cervical cancer patients in a worldwide study, in which HPV 16 had the strongest association with cervical cancer, whereas the low-risk types were rarely found among cervical cancer patients (Bosch *et al.,* 1995). HPV 16 together with the medium-risk group were equivalent to

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the ''oncogenic types'' defined in many other epidemiologic studies. If a subject was infected with multiple HPV types belonging to different risk categories, assignment to the higher-risk group took precedence.

Data analyses

In univariate analyses, prevalence or distribution of risk factors was compared among women with CIN I, II and III by χ^2 test, if the risk factor was a categorical variable, and by ANOVA and Wilcoxon rank sum test, if the risk factor was a continuous variable. In multivariate analyses, data were analyzed by the polytomous logistic regression method using Stata Statistical Software (StataCorp, 1997). In this multinomial logit model, the dependent variable had multiple categories (CIN I, II and III). By choosing CIN I as the base category for the dependent variable, the model estimated 2 risk equations simultaneously: the odds for having CIN II *vs.* CIN I and the odds for having CIN III *vs.* CIN I, each value being expressed as a function of covariates or independent variables. Therefore, 2 odds ratios (ORs) were estimated for a given risk factor from one maximum-likelihood model; the OR for having CIN II when individuals exposed to the risk factor were compared to the non-exposed and another OR for having CIN III. Because of skewed distributions, plasma levels of β-carotene and α-tocopherol were log-transformed. All *p* values presented are 2-tailed.

RESULTS

Among HPV-positive women with CIN, severity of CIN at entry into the study was significantly associated with age and educational level and exhibited a borderline association with ethnicity and frequency of Pap smear screening (Table I). These socio-behavioral variables could exert influence when CIN was detected during the course of natural history. For example, infrequent Pap smear screening may cause delay in CIN diagnosis and, hence, be associated with high-grade lesions, but it is not involved in the biological processes of CIN natural history. For the purpose of identifying biologically relevant risk factors associated with high-grade CIN, demographic and socio-behavioral variables were adjusted for in multivariate analyses.

Table II shows that HPV 16 was the predominant type in women with CIN II (37.3%) or CIN III (56.8%). Although several types (*e.g.*, uncharacterized, HPV 31, 58, 52 and 16) were found to have a relatively high prevalence in the CIN I group, no particular type(s) preponderated. Results from multivariate analyses (Table III) revealed that women infected with HPV 16 had a 12- to 24-fold increased risk of having high-grade lesions (*i.e.*, CIN II or III) compared with those with a low-risk type. Medium-risk types also tended to associate with high-grade CIN, though the association was not significant. To further evaluate the effects of HPV types, women were subcategorized by whether they were infected with a single type or multiple HPV types. Among women with single infection, those infected with an oncogenic type (HPV 16 or medium-risk type) were 5 to 6 times more likely to have high-grade lesions than those with a low-risk type. Among women with multiple types, those infected with oncogenic types only were at increased risk for high-grade lesions compared with those infected with at least one low-risk type [OR for CIN II = 3.67, 95% confidence interval (CI) = 1.24–10.91, $p = 0.019$; OR for CIN III = 7.03, 95% CI = 2.05–24.13, $p = 0.002$). Nevertheless, risk of high-grade lesions was comparable between women infected with multiple oncogenic types and those with only one oncogenic type. There was no association between high viral load and CIN grade at diagnosis (Table III).

Various life-style and sexual behavioral variables were also examined for a relationship with CIN grade at diagnosis in multivariate analyses adjusting for age, education, ethnicity, frequency of Pap smear screening and HPV type. CIN III, but not CIN II, was associated with current cigarette smoking and not using condoms during sex (Table IV). There was a dose-response relationship such that risk of CIN III increased with number of cigarettes smoked per day and number of pack-years of use, but this decreased with frequency of condom use. Smoking and condom use were independent risk factors as similar associations were observed when both variables were entered in a regression model. None of the following variables showed significant associations with CIN II or III: age at first coitus, number of male sexual partners in lifetime and last 12 months, frequency of vaginal sex and douching after sex, current and past use of oral contraceptive pills and number of pregnancies (data not shown).

TABLE I – DISTRIBUTION OF DEMOGRAPHIC FACTORS BY CIN GRADE AMONG HPV-POSITIVE WOMEN

	CIN I n (%)	CIN II n (%)	CIN III n (%)	*p* value
Age (years)				
<25	60 (36.8)	14 (27.5)	5 (11.4)	0.002[1]
25–34	64 (39.3)	22 (43.1)	22 (50.0)	
≥35	39 (23.9)	15 (29.4)	17 (38.6)	
Median age (interquartile range)	28 (22–34)	29 (24–35)	32 (27–39)	0.004
Ethnicity[2]				0.131
Black	66 (40.5)	28 (54.9)	15 (34.1)	
Hispanic	82 (50.3)	21 (41.2)	22 (50.0)	
Other	15 (9.2)	2 (3.9)	7 (15.9)	
Years of education				0.014
<12th grade	39 (24.1)	23 (45.1)	15 (34.1)	
≥12th grade	123 (75.9)	28 (54.9)	29 (65.9)	
Number of Pap smears in last 3 years				0.084[1]
0–2	43 (26.4)	17 (34.7)	17 (38.6)	
≥3	120 (73.6)	32 (65.3)	27 (61.4)	

[1]*p* for linear trend.–[2]*p* = 0.093 for comparison of frequencies of black and non-black among the 3 CIN groups.

TABLE II – TYPE-SPECIFIC HPV PREVALENCE IN PERCENTAGE BY CIN GRADE AMONG HPV-POSITIVE WOMEN[1]

HPV type	CIN I (n = 163)	CIN II (n = 51)	CIN III (n = 44)	All cases (n = 262)
6	8.6	2.0	0	5.7
16[2]	9.2	37.3(1)	56.8(1)	23.3(1)
18[2]	8.6	7.8	4.6	7.6
31[2]	10.4(2)	11.8(3)	2.3	9.2
33[2]	5.5	9.8	4.6	6.1
35[2]	4.9	5.9	6.8	5.3
39[2]	6.1	2.0	2.3	4.6
42	3.1	0	0	1.9
45[2]	4.9	3.9	4.6	4.6
51[2]	4.9	5.9	4.6	5.0
52[2]	9.2	13.7(2)	18.2(2)	11.8(2)
53	8.6	3.9	0	6.5
54	4.9	2.0	0	3.4
56[2]	8.6	11.8(3)	2.3	8.0
58[2]	9.8(3)	13.7(2)	11.4(3)	11.1(3)
59[2]	0	5.9	2.3	1.5
61	6.1	2.0	0	4.2
66	5.5	2.0	2.3	4.2
68[2]	3.7	0	0	2.3
70	2.5	7.8	0	3.1
73[2]	5.5	0	2.3	3.8
PAP291	2.5	3.9	2.3	3.1
AE7	3.7	2.0	2.3	3.1
AE8	2.5	5.9	2.3	3.1
Uncharacterized	12.3(1)	5.9	9.1	10.3

[1]Not presented here are types that were found in ≤5 subjects (types 11, 26, 32, 40, 55, 67, 69, 72, PAP155 and AE2). Superscript numbers in parentheses are the rank of the top 3 type-specific prevalences within each group.–[2]Oncogenic types that were found in a worldwide cervical cancer study (Bosch *et al.*, 1995).

TABLE III – CHARACTERISTICS OF GENITAL HPV INFECTION BY CIN GRADE AMONG HPV POSITIVE WOMEN

	CIN I n (%)	CIN II n (%)	CIN III n (%)	CIN II *vs.* CIN I		CIN III *vs.* CIN I	
				Adjusted OR (95% CI)[1]	*p* value	Adjusted OR (95% CI)[1]	*p* value
HPV type[2]							
Low-risk	56 (34.4)	7 (13.7)	5 (11.4)	1.0		1.0	
Medium-risk	92 (56.4)	25 (49.0)	14 (31.8)	2.10 (0.82–5.41)	0.123	1.87 (0.62–5.66)	0.265
HPV 16	15 (9.2)	19 (37.3)	25 (56.8)	11.96 (4.03–35.55)	<0.001	23.74 (7.39–76.26)	<0.001
Number of HPV types[3]							
Single	90 (55.2)	28 (54.9)	29 (65.9)	1.0		1.0	
Multiple	73 (44.8)	23 (45.1)	15 (34.1)	1.02 (0.52–1.99)	0.956	0.65 (0.32–1.33)	0.238
Combination of number of HPV types and specific types[3,4]							
Single infection with low-risk type	48 (29.4)	5 (9.8)[5]	5 (11.4)[5]	1.0		1.0	
Single infection with oncogenic type	42 (25.8)	23 (45.1)	24 (54.5)	5.47 (1.83–16.38)	0.002	6.21 (2.10–18.39)	0.001
Multiple infection with oncogenic types only	17 (10.4)	11 (21.6)	10 (22.7)	6.61 (1.92–22.77)	0.003	6.36 (1.83–22.06)	0.004
Multiple infection with at least one low-risk type[6]	56 (34.4)	12 (23.5)	5 (11.4)	2.02 (0.63–6.41)	0.234	0.93 (0.25–3.49)	0.919
Viral load							
Low	54 (33.1)	12 (23.5)	13 (30.2)	1.0		1.0	
High	109 (66.9)	39 (76.5)	30 (69.8)	1.68 (0.79–3.60)	0.180	1.25 (0.59–2.65)	0.556

[1]Odds ratio adjusted for age (continuous), education (<12 *vs.* ≥12 years), ethnicity (black *vs.* non-black) and number of Pap smears in last 3 years (<3 *vs.* ≥3).–[2]Medium-risk group: HPV types 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 and 73 (W13b was not detected in this study population). Low-risk group: all other types, excluding HPV 16 and medium-risk types.–[3]Individuals with uncharacterized type were assumed to have single infection in data analysis.–[4]Oncogenic type: HPV 16 or medium-risk type.–[5]All of these women with CIN II or III had uncharacterized HPV type.–[6]This group was not further divided into those who had both oncogenic and low-risk types and those who had low-risk types only because the latter had small numbers: 8 CIN I, 2 CIN II and 0 CIN III.

TABLE IV – LIFE-STYLE VARIABLES BY CIN GRADE AMONG HPV-POSITIVE WOMEN

	CIN I n (%)	CIN II n (%)	CIN III n (%)	CIN II *vs.* CIN I		CIN III *vs.* CIN I	
				Adjusted OR (95% CI)[1]	*p* value	Adjusted OR (95% CI)[1]	*p* value
Smoking status							
Never	102 (62.3)	27 (52.9)	18 (40.9)	1.0		1.0	
Ex-smoker	21 (13.0)	7 (13.7)	7 (15.9)	1.70 (0.60–4.81)	0.319	1.82 (0.64–5.22)	0.263
Smoker	39 (24.1)	17 (33.3)	19 (43.2)	1.46 (0.67–3.19)	0.337	2.37 (1.09–5.15)	0.030
Number of cigarettes per day							
Never or ex-smoker	123 (75.9)	34 (66.7)	25 (56.8)	1.0	0.478[2]	1.0	0.018[2]
≤10	27 (16.7)	12 (23.5)	9 (20.5)	1.30 (0.55–3.06)		1.49 (0.61–3.67)	
>10	12 (7.4)	5 (9.8)	10 (22.7)	1.38 (0.42–4.54)		3.35 (1.22–9.15)	
Number of cigarette pack-years							
Never	102 (63.0)	27 (52.9)	18 (40.9)	1.0	0.241[2]	1.0	0.019[2]
≤5	32 (19.8)	12 (23.5)	10 (22.7)	1.47 (0.62–3.48)		1.75 (0.71–4.31)	
>5	28 (17.3)	12 (23.5)	16 (36.4)	1.60 (0.67–3.83)		2.66 (1.15–6.15)	
Frequency of condom use in past year							
Never	58 (35.6)	19 (37.3)	26 (59.1)	1.0	0.462[2]	1.0	0.015[2]
Occasionally/sometimes	40 (24.5)	17 (33.3)	10 (22.7)	1.34 (0.56–3.21)		0.60 (0.24–1.50)	
Most/all of the time	65 (39.9)	15 (29.4)	8 (18.2)	0.73 (0.31–1.75)		0.32 (0.13–0.82)	

[1]Odds ratio adjusted for age, education, ethnicity, number of Pap smears in last 3 years and whether HPV infection was with an oncogenic type or not.–[2]*p* for linear trend.

When the effects of plasma micronutrient levels were examined, current smoking status was also entered into the multivariate model since cigarette smokers generally have decreased levels of plasma micronutrients (Basu *et al.,* 1990). Table V shows that levels of α-tocopherol and retinol did not correlate with the severity of CIN at diagnosis. High levels of β-carotene and reduced ascorbic acid appeared to be associated with CIN II and III, respectively, when these micronutrients were analyzed as continuous variables (Table V). Linear relationships, however, were not confirmed when these micronutrients were analyzed in quartiles as categorical variables (data not shown).

DISCUSSION

Our results show that HPV 16 and cigarette smoking are associated with high-grade lesions, particularly CIN III. These factors may predispose a woman to rapid development of high-grade instead of low-grade lesions, or they may increase the risk of progression of pre-existing low-grade lesions to high-grade lesions.

This and previous studies have shown that women infected with oncogenic HPV types, particularly HPV 16, are susceptible to high-grade CIN (Koutsky *et al.,* 1992; Schiffman *et al.,* 1993). Oncogenic HPV types increase the chance of persistent HPV infection (Ho *et al.,* 1998*a*), which in turn may cause high-grade CIN directly (Koutsky *et al.,* 1992). Viral chronicity may also allow accumulation of cellular and chromosomal damage brought on by additional cofactors and carcinogens, which leads to progression of low-grade to high-grade CIN (Ho *et al.,* 1995; Romney *et al.,* 1997).

A high viral load and infection with multiple oncogenic types, however, do not augment the risk for high-grade CIN, as demon-

TABLE V – MEAN PLASMA MICRONUTRIENT LEVELS BY CIN GRADE AMONG HPV-POSITIVE WOMEN

	CIN I Mean (SD)	CIN II Mean (SD)	CIN III Mean (SD)	CIN II *vs.* I		CIN III *vs.* I	
				Adjusted OR (95% CI)[1]	*p* value	Adjusted OR (95% CI)[1]	*p* value
Reduced ascorbic acid (mg/dl)	0.46 (0.25)	0.44 (0.21)	0.48 (0.24)	1.00 (0.21–4.77)	1.000	2.86 (0.61–13.52)	0.184
Log α-tocopherol (mg/l)	0.86 (0.14)	0.86 (0.14)	0.85 (0.17)	1.68 (0.12–23.16)	0.699	0.63 (0.04–9.01)	0.733
Log β-carotene (mg/dl)	1.11 (0.29)	1.20 (0.33)	1.04 (0.30)	4.56 (1.06–19.53)	0.041	0.49 (0.13–1.82)	0.289
Retinol (mg/dl)	63.59 (18.54)	60.70 (19.75)	66.39 (23.60)	1.01 (0.99–1.02)	0.521	1.01 (1.00–1.03)	0.124

[1]Odds ratio for a unit increase in micronutrient level adjusted for age, education, ethnicity, number of Pap smears in last 3 years, whether HPV infection was with an oncogenic type or not and current smoking status.

strated by our data. Integration of HPV DNA into the host genome, which enhances expression of the E6 and E7 oncoproteins and often disrupts the *E2* and *E1* genes for viral replication, occurs frequently in cervical cancer and some cases of CIN III. This suggests that expression of oncoproteins may be more important than a productive viral replication (*i.e.*, a high viral load) for establishment of high-grade lesions and neoplastic progression (Thierry, 1996).

Impairment of the host tumor-suppressor genes by HPV oncoproteins may render host cells susceptible to insults by other carcinogens (Palefsky and Holly, 1995). Previous case-control studies linked cigarette smoking with the etiology of CIN III and cervical cancer (Becker *et al.,* 1994; Ngelangel *et al.,* 1998; Schiffman *et al.,* 1993). Here, we demonstrate an association between cigarette smoking and CIN III, but not CIN II, in women with HPV infection. The data suggest that cigarette carcinogens may be involved in the later stages of the natural history of HPV-associated CIN. These carcinogens may be responsible for inducing genomic damage and driving HPV-infected cells toward tumorigenesis. This hypothesis is supported by several findings among smokers: detection of mutagenic cervical fluids (Holly *et al.,* 1986), high concentration of nicotine and tobacco-specific *N*-nitrosamines in cervical mucus (Prokopczyk *et al.,* 1997) and increased levels of DNA adduct in cervical epithelium (Simons *et al.,* 1995). Although it has been suggested that smoking induces suppression of local immune response and facilitates persistent HPV infection (Palefsky and Holly, 1995), smoking has been reported to be a protective factor for type-specific persistent HPV infection in 2 prospective studies (Hildesheim *et al.,* 1994; Ho *et al.,* 1998*a*).

The question remains whether the effects of cigarette smoking are dependent on HPV types such that cigarette carcinogens increase the likelihood of high-grade CIN only if the CIN lesions are associated with oncogenic HPV types and have no effects on lesions associated with the low-risk HPV types. This interaction could not be examined in our study due to the small number of high-grade CIN cases infected with the low-risk HPV types.

The association between CIN III and infrequent use of condoms is intriguing and may be due to some confounding factors that were not measured. As part of this study, we have also found the presence of antibodies to *Chlamydia* to be a risk factor for CIN III; however, positive serology to *Chlamydia* and condom use appeared to have independent associations with CIN III, as shown in multivariate analysis (data not shown). Therefore, infrequent condom use may not be a proxy for infection with other sexually transmitted agents.

When the CIN cases in our study were compared with control women without a history of abnormal Pap smears, low plasma levels of reduced ascorbic acid and α-tocopherol were significant risk factors for the development of CIN (Ho *et al.,* 1998*b*). Further analyses among the CIN cases, however, did not find HPV-positive women with low-grade lesions to have a better plasma micronutrient profile than those with high-grade lesions. Another prospective study also did not identify a relationship between plasma micronutrients, regression of CIN and resolution of HPV infection (Romney *et al.,* 1997). It is possible that anti-oxidants act as first-line defenses against initial acquisition of HPV infection and CIN development and have little effect on the outcome of CIN once the infection and lesion have been established.

Genital HPV infection, particularly infection with HPV 16 and/or other oncogenic types, is the major risk factor for the development of CIN, and it also has a significant impact on the natural history of cervical neoplasia. Oncogenic HPV types induce viral chronicity and continuous expression of the E6 and E7 oncoproteins, which impair function of host tumor-suppressor genes (Palefsky and Holly, 1995). Host immunity may interact, resulting in resolution of HPV infection. However, if the state of viral chronicity persists, it may allow host genetic changes initiated by mutagens, such as those in tobacco, to accumulate. The lesion may eventually exhibit the morphology of high-grade CIN III with chromosomal abnormalities and subsequently progress to cervical cancer. If cigarette smoking is confirmed in prospective studies to be a significant cofactor in the neoplastic progression of CIN, smoking cessation should then be considered as a means of prevention and control of cervical cancer.

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