# Spontaneous Regression of High-Grade Cervical Dysplasia: Effects of Human Papillomavirus Type and HLA Phenotype

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# Spontaneous Regression of High-Grade Cervical Dysplasia: Effects of Human Papillomavirus Type and HLA Phenotype

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**Abstract**

**Purpose:** Persistent infection with oncogenic human papillomaviruses (HPV) plays a central etiologic role in the development of squamous carcinomas of the cervix and their precursor lesions, cervical intraepithelial neoplasias (CIN). We carried out a prospective observational cohort study evaluating known, quantifiable prognostic variables of clinical behavior in women with high-grade cervical lesions.

**Experimental Design:** Our study cohort included healthy women with high-grade cervical lesions (CIN2/3) with residual visible lesions after colposcopically directed biopsy. We prospectively followed 100 women over 15 weeks before standard resection. HPV typing was done using PCR and a reverse line blot detection method.

**Results:** The rate of spontaneous histologic regression, defined as (CIN1 or less at resection) was 28%. The overall rate of HPV infection was 100%. HPV16 was identified in 68% of the lesions. Women with HPV16 only were significantly less likely to regress, compared with women with HPV types other than HPV16 (odds ratio, 0.342; 95% confidence interval, 0.117-0.997; $P = 0.049$). In the cohort with HPV16 only, patients who had an *HLA*A201* allele had similar outcomes to those who did not carry A201. However, among patients with HPV types other than HPV16, the *HLA*A201* allele interaction was significant; patients with *HLA*A201* were the least likely to resolve.

**Conclusions:** CIN2/3 lesions associated with HPV16 alone are significantly less likely to resolve spontaneously than those caused by other types. Interactions among HPV type, HLA type, and regression rate support a role for HLA-restricted HPV-specific immune responses in determining disease outcome.

Persistent infection with a high risk, or oncogenic type of human papillomavirus (HPV) is necessary but not sufficient for the development of most squamous carcinomas of the cervix and their precursor lesions, cervical intraepithelial neoplasia (CIN; ref. 1). CIN1, CIN2, and CIN3 lesions represent a spectrum of disease. Low-grade, or CIN1 lesions, represent a chronic HPV infection, in which HPV DNA is episomal and intact virion production and shedding occur. In women who are immunocompetent, many low-grade, or CIN1 lesions, will nonetheless eventually regress without intervention (1, 2). Reported rates of regression range up to 58% over 24 months (3). A very small percentage (~2%) will progress to high-grade lesions.

In contrast, most high-grade, or CIN2/3 lesions are thought to be much more likely to persist than to regress. However, reported rates of spontaneous regression vary from 6% to 50%, depending on diagnostic criteria, and length of follow-up (4). The risk for progression to invasive cancer at 24 months in women with high-grade lesions is ~1% to 2%.

The mechanisms by which HPV-associated intraepithelial lesions resolve are not well understood. As a prelude to interventional clinical trials in women with biopsy-proven CIN2/3, we carried out a prospective observational cohort study evaluating known, quantifiable prognostic variables in immune competent women with biopsy confirmed CIN2/3 over an observation window before routine therapeutic excision. Our purpose was to estimate rates of spontaneous regression after diagnostic biopsy in immune competent women and to investigate clinical, immunologic, and virologic differences between women whose disease regressed and women whose disease did not. The careful characterization of lesions that are likely to regress without intervention is critical to the design of interventional trials in this patient population.

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## Materials and Methods

***Study design.*** We prospectively invited women who had been referred to the Johns Hopkins Colposcopy Service for evaluation of a high-grade Papanicolaou (Pap) smear to participate in an observational study protocol before standard therapeutic surgical resection of histologically confirmed high-grade cervical lesions. All women referred for evaluation of a high-grade Pap smear were approached, unless they were (a) pregnant, (b) immunocompromised, or (c) known to have active substance abuse precluding compliance with follow-up. Patients evaluated in this analysis had CIN2/3 confirmed by colposcopically directed biopsy and had a visible lesion after the diagnostic biopsy. We chose histologic diagnosis of CIN2/3 as our entry criterion as opposed to cytologic diagnosis, because the reproducibility of histologic diagnoses of CIN2/3 is significantly more robust than the reproducibility of cytologic diagnoses of high-grade lesions (5, 6).

Subjects were asked routine questions about demographic and behavioral factors, including reported onset of sexual activity, number of lifetime partners, history of sexually transmitted diseases, tobacco smoking, and contraceptive use. The primary outcome of this analysis was persistence of CIN2/3 versus regression, defined as CIN1 or less, at resection at 15 weeks.

Subjects underwent a single interval visual colposcopic inspection at week 8. At this visit, a cervical swab was obtained for HPV typing. At the time of therapeutic cone excision of the transformation zone at week 15, a third cervical swab was obtained for HPV typing and colpographs were obtained.

In the period from March 2000 to June 2004, a total of 187 subjects referred for evaluation of a high-grade Pap smear were recruited for screening for study eligibility. Of women who signed consent for screening, 65 of 187 (34.8%) did not have biopsy-confirmed CIN2/3. This figure corresponds with the positive predictive value of cytologic screening diagnoses found in large trials (5, 6). Common diagnoses in women with high-grade Pap referrals who did not have biopsy-confirmed lesions included low-grade lesions, atrophy, and atypical immature metaplasia. Other reasons for nonenrollment included interval pregnancy ($n = 3$), diagnosis of adenocarcinoma *in situ* at biopsy ($n = 2$) noncompliance ($n = 16$), and interval myocardial infarction ($n = 1$).

To date, a total of 100 women with biopsy-confirmed CIN2/3 have completed follow-up with end point resection. All colposcopic examinations were done by a single gynecologist (C.L.T.). A diagnostic biopsy was obtained from the most abnormal sites at the initial visit, and a cervical swab was obtained for HPV typing. Colposcopy was done by placing a bivalve speculum in the vagina and directly visualizing the cervix. A dilute acetic acid wash (1%) was applied to the cervix, and green filter light used to identify acetowhite lesions and abnormal vascular patterns. A single punch biopsy of the most abnormal area was done. Colposcopy was done again immediately before therapeutic tissue resection. Histologic specimens were read routinely. Histologic grading of dysplasia was based on standard CIN1, CIN2, and CIN3 criteria. All histologic material was re-reviewed by a second gynecologic pathologist in a manner blinded to subject identity and clinical history. HPV typing was detected in cervical swab samples using PCR and a reverse line blot assay (7). HLA typing was done on peripheral blood lymphocytes through the Johns Hopkins Immunogenetics Core Lab. HLA alleles were identified by hybridization of sequence-specific oligonucleotide probes to human genomic DNA extracted from whole blood. High resolution sequence-based typing was done for HLA-A locus exons 2, 3, and 4; HLA-B locus 2, 3, and 4; and Cw locus 2 and 3. Intermediate resolution typing was carried on for HLA class II loci *DRB1* and *DQB1*.

***Human papillomavirus typing: Sample processing.*** One hundred–microliter aliquots of the original swab sample in Digene standard transport medium were removed and added to 10 μL of a 10× digestion buffer [1 mol/L Tris-HCl (pH 7.5), 0.5 mol/L EDTA, 1% Laureth-12, and 4 mg/mL proteinase K]. After vortexing, the sample + digestion buffer was incubated at 65°C for 1 hour. Following heat inactivation of the protease at 95°C for 10 minutes, the digest was mixed with cold precipitation solution (0.825 mol/L ammonium acetate and 83.5% ethanol) and the DNA was precipitated overnight at −30°C. DNA was pelleted at 15,000 rpm for 30 minutes at 4°C, supernatant removed and the pellet dried for 30 minutes at 42°C. Samples were resuspended in 50 μL LoTE [1 mmol/L Tris + 0.5 mmol/L EDTA (pH 7.5)]. Extraction controls were included in each processing batch of 30 to 40 samples. These included a high extraction control comprised of $1.25 \times 10^5$ SiHa cells and $1.0 \times 10^6$ K562 cells per mL standard transport medium; a low extraction control comprised of $5.0 \times 10^3$ SiHa cells and $1.0 \times 10^6$ K562 cells per mL standard transport medium; and a negative extraction control comprised of $1.0 \times 10^6$ cells per mL standard transport medium. A 100-μL aliquot of each extraction control was processed with each batch.

***Human papillomavirus genotyping.*** A 5-μL aliquot of each DNA sample was amplified using PGMY09/11 L1 consensus primers, and genotype discrimination of 27 common genital HPV types was done using reverse line blot hybridization as previously described (7, 8). The PGMY/linear array reagents were a kind gift from Roche Molecular Systems, Inc. (Pleasanton, CA).

***HPV16 quantitation.*** All assays were done using the ABI 5700 Sequence Detection System. HPV16 was quantified using Taqman PCR methods as described previously (9). Essentially, 2.5 μL of extracted DNA, control DNA, or water (as no template control) were added to 47.5 μL of master mix containing 1× PCR buffer [10 mmol/L Tris-HCl (pH 8.0) + 50 mmol/L KCl]; 200 μmol/L each dATP, dGTP, dCTP, and dTTP; 0.1 μmol/L Taqman probe; 0.2 μmol/L each primer; 4 mmol/L $MgCl_2$; and 5 units of AmpliTaq Gold DNA polymerase. Samples were amplified using the following thermal profile: 50°C for 2 minutes, 95°C for 12 minutes, and 50 cycles of 95°C for 15 seconds and 55°C for 30 seconds. Following amplification, the ABI 5700 Sequence Detection System detection software is employed by manual selection of threshold based on observed growth curves.

We also sought to control for sampling variability by normalizing the HPV16 viral load to total human DNA processed. Using a 2.5-μL aliquot of each sample, we also amplified using Taqman real-time PCR techniques an endogenous human retrovirus (ERV-3), presumed to be present at two copies per diploid cell (10). In brief, 2.5 μL of sample DNA were added to 47.5 μL of master mix containing 1× PCR buffer [10 mmol/L Tris-HCl (pH 8.0) + 50 mmol/L KCl]; 200 μmol/L each dATP, dGTP, dCTP, and dTTP; 0.25 μmol/L Taqman probe; 0.4 μmol/L each primer; 4 mmol/L $MgCl_2$; and 5 units of AmpliTaq Gold DNA polymerase. Samples were amplified using the following thermal profile: 95°C for 10 minutes and 50 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

Both the HPV16 and the ERV-3 assays quantified unknown samples based on an external standard curve amplified in each assay. For HPV16, this standard curve was generated from a dilution series of HPV16 plasmid in a background of 50 ng/μL human placental DNA. For ERV-3, purified human DNA from the diploid human lung cell line CCD-18Lu (ATCC CCL 205) was diluted serially into a background of 50 ng/μL of salmon sperm DNA (Invitrogen, San Diego, CA). The slope and intercept from a log-linear graph of standard copy number and $C_t$ were used to estimate the unknown copy number based on the measured $C_t$ for the each unknown. Normalized viral load is presented as total HPV16 copy number per 1,000 cell equivalents (ERV-3 copies/2 to account for diploid genome).

This protocol was approved by the Johns Hopkins Hospital Institutional Review Board. Informed consent was obtained from each patient. These investigations were done in accordance with the principles embodied in the Declaration of Helsinki.

***Statistical methods.*** The primary statistical outcome of the study was spontaneous regression of lesions, defined as CIN1 or less at 15 weeks. We compared characteristics of women who had spontaneous

regression of their lesions over the study period with those who had persistent disease. The outcome of CIN at resection was taken as a dichotomous variable. Its univariate association with other categorical factors (e.g., HPV typing) was assessed in contingency tables, by estimated odds ratios (OR), and by the $\chi^2$ statistic. Continuously distributed predictors in patients who regressed versus those who did not (e.g., age) were compared using means and *t* tests. To account for the effects of more than one predictor simultaneously on the outcome, multivariable logistic regression models were used. In all cases, the outcome was regression of the lesion versus persistence. All models included time measured from diagnosis to surgical resection in months as a predictor. To test predictor variables for their association with outcome, a backward elimination stepwise procedure was used for model building. All candidate variables were entered into the model, and statistically nonsignificant (and/or clinically weak) factors were removed from the model one at a time, with reestimation of ORs, confidence intervals (CI), and *P* values at each step. All proportions are given with 95% exact binomial confidence limits.

## Results

***Patient characteristics.*** Subjects were recruited an average of 1 month after referral from their index abnormal Pap smear diagnosis, at their initial referral visit for colposcopic evaluation of an abnormal pap smear. The clinical characteristics of the 100 subjects with a complete diagnostic biopsy/therapeutic resection tissue set are shown in Table 1. The average age was 29.7 years (median, 26.5 years; range, 18-67 years). Mean parity was 1.76 (range, 0-11). Mean number of reported lifetime sexual partners was 8.1 (range, 1-50). The ethnic distribution included 26 (26%) African American, three (3%) Hispanic, 67 (67%) White, and four (4%) Asian. A reported history of current tobacco smoking was obtained from 42 (42%). The average interval from biopsy to resection was 123.8 days (median, 110.0 days). This cohort reflects the ethnic and demographic distribution of women with HPV disease of the lower genital tract referred to our institution.

***Histologic outcomes.*** We compared clinical, histologic, and virologic characteristics in this cohort of 100 women (Table 2). The overall rate of spontaneous histologic regression, defined as CIN1 or less at the time of conization, was 28%. There was no significant difference in age, time to resection, or use of hormonal contraceptives in women whose disease regressed compared with those whose disease did not. The current use of tobacco was slightly but not significantly more likely in women with persistent disease than in women whose disease regressed.

**Table 1.** Clinical characteristics of women with CIN2/3

| | |
|---|---|
| Median age (y) 30.1 y; range, 18-67 y (%) | |
| <25 | 25 (25) |
| 25-34 | 53 (53) |
| >35 | 22 (22) |
| Average time to resection | 123.8 d |
| Ethnicity (%) | |
| African American | 26 (26) |
| Hispanic | 3 (3) |
| White | 67 (67) |
| Asian | 4 (4) |
| Reported number of partners | 8.1 (1-50) |
| Tobacco smoking (%) | |
| Current | |
| Former | 2 (2) |
| Never | 56 (56) |
| Hormonal contraceptive use (%) | 52 (52) |

**Table 2.** Patient characteristics: spontaneous regression versus persistent disease

| Patient characteristics | Persistent disease (%) | Regress disease (%) | *P* |
|---|---|---|---|
| *N* | 72 (72) | 28 (28) | |
| Current tobacco use (*n* = 42) | 26/42 (61.9) | 16/42 (38.1) | 0.21 |
| Hormonal birth control (*n* = 52) | 39/52 (75) | 13/52 (25) | 0.95 |
| Age > 25 (*n* = 75) | 58/75 (77.3) | 17/75 (22.7) | 0.06 |
| HLA*A201, yes (*n* = 30) | 23/30 (76.7) | 7/30 (23.3) | 0.41 |

***Human papillomavirus typing.*** The prevalence of HPV in this cohort was 100% and 98% for high-risk types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and W13b). HPV16 was found in 68%. Either HPV16 or other HPV16-related types (31, 33, 35, 52, and 58) were found in 81 of 100 (81%).

In a subset of the cohort that had HPV16 infection at study entry (*n* = 52), we found that coinfection with other types at study entry was common (33%). We also found that new infections over the observation period were common (32.7%) and that clearing HPV types over the observation period was also common (38.5%; data not shown).

Quantitation of HPV16 viral load in these patients in longitudinal specimens suggested a threshold effect. Patients whose quantitative viral load had dropped below 1,000 copies per 1,000 cell equivalents by their week 15 visit, in general, resolved their lesions. In contrast, patients whose viral load increased over the observation period, particularly to high levels, did not clear their lesions (Fig. 1; quantitative HPV16 viral load over time).

In the study window of observation, spontaneous regression occurred in 9 of 44 (20.5%) of those women with single infection with HPV16 (Table 3). In contrast, in women infected with types other than type 16, the rate of spontaneous regression was 12 of 33 (36.4%). In women infected with both HPV16 and at least one other HPV type, spontaneous regression occurred in 7 of 24 (29.2%). Overall, the effect of single-type infection with HPV16 alone conferred a 3-fold reduction in the likelihood of disease regression over the study window compared with women who had lesions not associated with HPV16 (OR, 0.34; 95% CI, 0.117-0.997; *P* = 0.05; Table 4).

The rate of lesion regression in patients who had infections with HPV16-related types (31, 33, 35, 52, and 58; 26%) was very close to that which we observed in the HPV16-only group. In contrast, the rate of regression in patients who had HPV infections that were not HPV16 or HPV16 related was 61.5%. Lesions not associated with HPV16 or HPV16-related types were significantly more likely to resolve than lesions associated with HPV16 (*P* = 0.04).

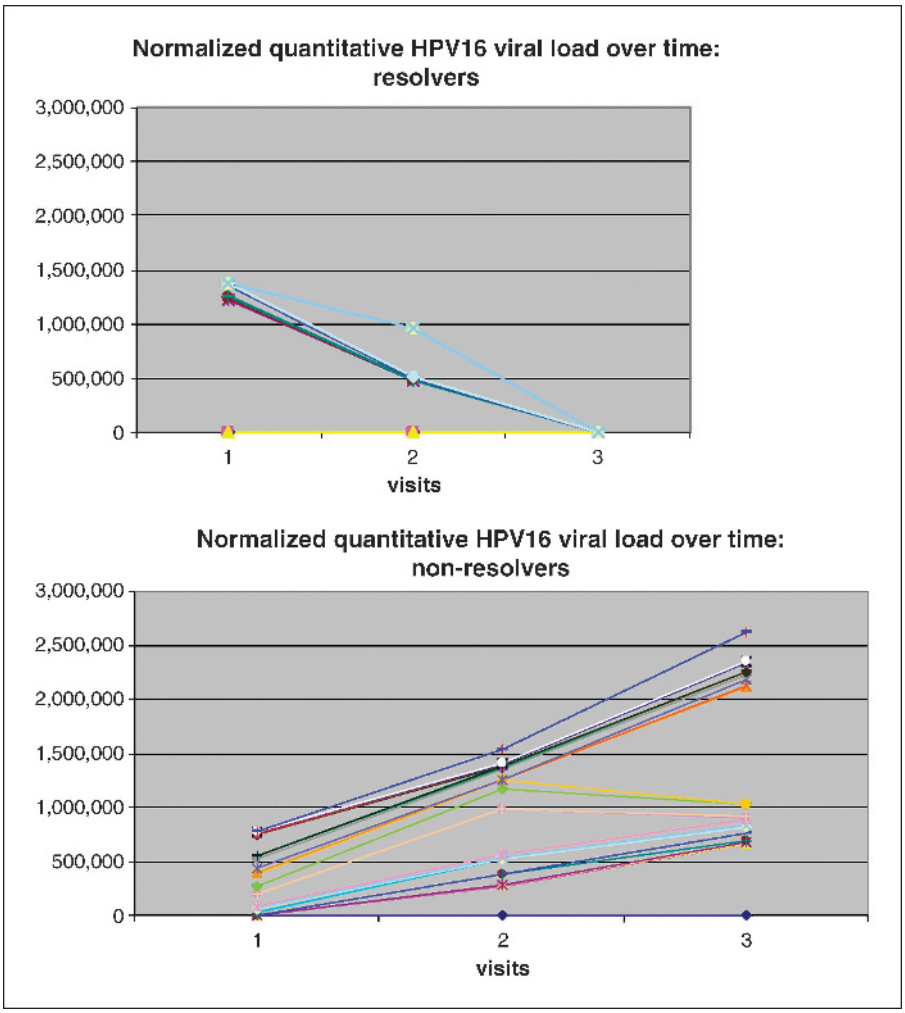


**Fig. 1.** Serial cervical swabs were obtained from patients with HPV16-associated lesions. Visit 1, screening visit; visit 2, week 8; visit 3, week 15 (therapeutic resection of transformation zone). Using qunatitative PCR and primers for HPV16E6 and normalizing to ERV-3, serial cervical swabs were assessed. Quantitative HPV16 viral load on the cervix over time in patients whose lesions resolved spontaneously over the 15-week observational window (*top*). Patients whose lesions did not regress (*bottom*).

***HLA typing.*** HLA class I typing was done on this cohort. The distribution of alleles was similar to that seen in the general population. In our study population, 30% of the patients carried an *HLA*A201* allele. In the women who had single-type infection with HPV16 only, the presence of an *HLA*A201* allele did not have significant effect on rates of regression. In contrast, in the group of patients with infections with HPV types other than HPV16, there was significant interaction with *HLA*A201*. Specifically, women with non-HPV16 high-grade lesions who carried an *HLA*A201* allele were 3-fold less likely to resolve their lesions than those who did not have *HLA*A201* (14.3% versus 42.3%). We investigated the interaction between the presence or absence of HPV16 and *HLA*A201* using a multivariable logistic model, adjusted for time from diagnosis to surgical resection (Table 4). The *HLA*A201* allele was negatively associated with lesion regression in women with lesions caused by HPV types other than type 16 or HPV16-associated types (OR, 0.03; 95% CI, <0.001 to 1.034; $P = 0.05$).

**Table 3.** Patterns of HPV infection: spontaneous regression versus persistent disease

| | Persist (%) | Regress (%) | *P* |
|---|---|---|---|
| HPV16 alone ($n = 44$) | 35/44 (79.5) | 9/44 (20.5) | 0.05 |
| HPV16 plus others ($n = 24$) | 17/24 (70.8) | 7/24 (29.2) | 0.08 |
| Other HPV, not HPV16 ($n = 32$) | 19/32 (59.4) | 13/32 (40.6) | 0.08 |
| Not HPV16, not HPV16-related (13/32) | 5/13 (38.5) | 8/13 (61.5) | |
| Total ($n = 100$) | 72/100 (72) | 28/100 (28) | |

## Discussion

In this analysis, we have compared clinical, virologic, and histologic characteristics of patients with biopsy-confirmed CIN2/3 over a short observational window before standard therapeutic resection. We found an overall spontaneous regression rate of 28% over a 15-week window. Women with CIN2/3 associated with infection with HPV16 were significantly less likely to have lesion regression compared with the rest of the cohort. However, HPV16-positive patients who resolved their lesions in general had lower HPV16 viral loads than those who failed to resolve their lesions. Moreover, those who did resolve their lesions had decreasing viral loads over the observation window, in contrast to patients who did not

**Table 4.** Multivariable logistic regression for lesion resolution

| Variable | Estimated OR (95% CI) | *P* |
|---|---|---|
| Months | 0.99 (0.84-1.16) | 0.86 |
| HPV16 | 0.34 (0.12-0.99) | 0.05 |
| Months | 0.99 (0.84-1.16) | 0.86 |
| HPV16 | 0.35 (0.12-1.01) | 0.05 |
| HLA*A201 | 0.71 (0.25-1.99) | 0.52 |
| Months | 1.01 (0.86-1.19) | 0.87 |
| HPV16 | 0.20 (0.06-0.73) | 0.01 |
| HLA*A201 | 0.90 (0.03-29.44) | 0.95 |
| Non-HPV16xHLA*A201 | 0.03 (<0.001-1.03) | 0.05 |

resolve their lesions. Finally, the interaction of host HLA class I type with the HPV types identified increased the effect of the HPV infection by an order of magnitude, supporting the hypothesis that the efficacy of MHC class I–restricted presentation of viral antigens plays an important role in disease outcome.

The strengths of this study include its prospective design, histologic diagnosis of CIN2/3 as an entry criterion, virologic data, and the standardization of colposcopy and histopathologic review. However, the relatively small sample size ($n$ = 100) as well as our incomplete understanding of the potential effect of biopsy on lesion behavior limit interpretation of our data.

The effect of a diagnostic biopsy on the natural history of CIN2/3 may explain the discrepancy between our observations and those of several large studies based on cytologic diagnosis alone, as the local response to a diagnostic biopsy may be quite different than a response to a cytologic smear, which is atraumatic and noninvasive. HPV infects the basal cell layer of the cervical mucosal squamous epithelium without disruption of the basement membrane. Subsequent production of foreign or viral proteins occurs with cellular maturation and takes place in the most superficial layer of epithelium. Viral antigens are thus released in the setting of natural epithelial senescence and desquamation and are therefore not necessarily presented to host immune system in the context of signal two or danger. Therefore, aside from decreasing lesion size, a diagnostic biopsy may elicit enough of a local reparative or inflammatory milieu so that viral antigens previously undetected may be presented in a setting sufficient to induce an effective immune response and subsequent lesion clearance.

Holowaty et al., for example, found a regression rate of 6.9% over 2 years in a large cohort study of women with moderate dysplasia (2). This study was based on cytologic diagnoses only and was not linked with diagnostic biopsies. In our patient cohort, 65 of 187 (34.8%) of the women referred for evaluation of high-grade cytology did not have histologically confirmed high-grade lesions. Narod et al. followed a cohort of 70,236 women, also using cytologic diagnoses for an end point of progression to carcinoma *in situ* or worse (11). Again, as there was no histologic confirmation, it is difficult to extrapolate these findings to compare their clinical significance with the findings in our cohort.

The observed rate of spontaneous regression of biopsied CIN2/3 in this cohort is within the range of that seen in control/placebo arms of published interventional clinical trials in similar patient cohorts (Table 5; refs. 12–14). However, none of these studies included HPV type–specific correlation.

Previous studies have assessed risk for incident intraepithelial lesions in the setting of persistent HPV infections (15–17). Duration of infection with HPV type 16 in particular has been shown to be longer than with other HPV types (1). Ho et al. evaluated the natural history of HPV infection in a cohort of college women. These authors found that multiple infection conferred a 4-fold increase in risk for persistent HPV infection at >6 months. However, this effect was noted in women without incident lesions. They found that persistent infection with the same HPV types, particularly high-risk types, conferred the highest risk for subsequent incident intraepithelial lesions. The association in our cohort between women with single infection with HPV16 and persistent established high-grade lesions may reflect an inability on the part of these women to have resolved a persistent HPV16 infection in the first place. Coinfection with other HPV types may elicit an immune response capable of resolving an established lesion regardless of the HPV type causing it.

We made the assumption that in those patients infected with one HPV subtype only, the CIN2/3 arose from that HPV subtype. Integration of viral DNA into the host cell genome is a rare event; high-grade HPV cervical lesions are thought to be clonal processes (18, 19). Our data is consistent with earlier reports documenting infections with multiple types of HPV among both women with normal cytology and those with histologic CIN (20–23). In the ALTS study, for example, Sherman et al. have reported that ~50% of CIN2 and CIN3 lesions were associated with multiple oncogenic HPV types. Our study suggests that HPV16 infection, compared with other

**Table 5.** Clinical behavior of biopsy-confirmed CIN2/3

| Author | Sample size ($n$) | Patient population | Time interval | Rate of regression (%) |
|---|---|---|---|---|
| Follen (2001) | 17 | CIN2/3 | 12 mos | 50 |
| Meyskens (1994) | 48 | CIN2 | (21-27 mos) | 27 |
| Meyskens (1994) | 35 | CIN3 | (21-27 mos) | 31 |
| Keefe (2001) | 20 | CIN2 | 24 mos | 19 |
| | 32 | CIN3 | 24 mos | 19 |
| Alvarez (2003) | 38 | CIN2/3 | 12 wks | 32 |
| Trimble | 100 | CIN2/3 | 15 wks | 28 |

HPV types, continues to confer an adverse effect in the setting of an established high-grade lesion.

A classic approach to determine whether a disease outcome has an immunologic component is to search for associations with specific HLA alleles. We therefore initially assessed whether natural regression was affected by the presence or absence of the most common HLA class I allele (*A201*) expressed by roughly half of the population. We found that, whereas HLA-A201 expression had relatively little effect on regression in HPV16+ lesions, it displayed a pronounced effect in non-HPV16 lesions. HLA-A2+ individuals with non-HPV16+ lesions were 30-fold less likely to experience natural regression than HLA-A2− patients with HPV16. The specific role of MHC class I–restricted immune responses in natural regression of established but premalignant cervical HPV lesions remains to be elucidated. The simplest mechanism is an "Ir gene" mechanism in which certain HLA alleles fail to present peptides efficiently. The relatively small size of E6 and E7 proteins might indeed provide a limited number of epitopes available for presentation. Application of this model to the current data set would suggest that evolutionary pressure on the less oncogenic HPV types (i.e., non-HPV16) would have been to eliminate E6 and E7 epitopes efficiently presented by common HLA alleles such as *HLA-A201*. Whereas reports of association between cervical HPV disease and HLA haplotypes have not been consistent, an increasing body of evidence supports increasing down-modulation of the MHC class I antigen presentation machinery with progression of premalignant lesions. Both HLA (I and II) allele association and abnormal HLA class I antigen presentation have been reported in other chronic human viral infections such as hepatitis B and C (24, 25). Discrete HLA supertypes have been shown to be associated with differential clinical responses to HIV infection (26). Taken together, the emerging data on HLA allele association and down-modulation of antigen presentation provide evidence for a T-cell recognition component to the outcome of HPV-associated premalignant cervical disease.

Other immunologic mechanisms must also be considered besides a straightforward HLA-restricted antigen presentation model. In fact, an Ir gene mechanism predicts that a failed response (i.e., lack of regression) is recessive. However, the majority of HLA-A201 patients are heterozygous at this locus, suggesting that the decreased rate of regression in HLA-A201+ individuals with non-HPV16 lesions is due to a dominant effect. This dominant effect could be related to the generation of regulatory T cells. Regulatory T cells are typically thought to be CD4+ and would thus not recognize HLA-A201; however, CD8+ regulatory T cells have recently been described (27–29). Clearly, it will be important to identify the HPV epitopes which are naturally processed in patients and to generate T-cell lines and clones grown from regressing and nonregressing patients. Finally, a role for HLA allele expression on natural killer responses to HPV-infected cells must also be considered, owing to the ability of certain class I alleles to inhibit natural killer responses via killer inhibitory receptors of the immunoglobulin superfamily. Matching between specific HLA C alleles and killer inhibitory receptor haplotypes has been recently reported to affect the outcome of HCV infection (30). The role of natural killer responses in controlling premalignant HPV lesions has not yet been assessed but warrants serious study. The direct accessibility of HPV+ cervical lesions offers unique opportunities to analyze the phenotype and function of lymphocyte populations directly infiltrating regressing and nonregressing lesions.

Whereas fluctuations in the local humoral immunologic milieu in the cervix fluctuates with the menstrual cycle, pregnancy, and use of hormonal contraceptives have been described (31, 32), we did not observe any effect of current oral contraceptive use upon rate of spontaneous regression. As noted above, however, our sample size is relatively small and the 15-week window of observation is very short; therefore, only large effects would be obvious.

Tobacco smoking is known to increase the risk of cervical dysplasia, in a dose-response fashion, from low-grade lesions to frank invasive cancer (15, 33–39). Nicotine and nicotine metabolites have been found in cervical secretions of women who smoke (40–43). We observed a nonsignificant trend in our cohort toward an increased risk for persistent disease among women who were current smokers.

The length of observation in this study was motivated by concern over patient safety. The risks of progression to invasive disease in the time period chosen (15 weeks) were believed to be virtually zero. Based on our observations, as well as those of others, an argument for a longer time period of study design could be made (12, 13, 44). Nonetheless, such studies mandate careful patient selection, regular colposcopic follow-up, and aggressive efforts to ensure that patients are not lost to follow-up before definitive therapy has been done.

Appropriate intermediate end points are critical to the design of interventional trials in patients with preinvasive disease. To date, only cytologic and histopathologic diagnoses have been sufficiently validated to warrant use as end points (45). The use of other biological markers and emerging noninvasive imaging technologies remain experimental. To be useful as potential intermediate end points, they must be quantifiable, reproducible, and be shown to correlate with known biological variables of HPV disease. We found that quantitative HPV16 viral load in cervical swabs correlated with clinical behavior. This measure should be further evaluated in future interventional trials. For the immediate future, colposcopically directed tissue biopsy remains the gold standard end point for design and analysis of interventional trials in this population of women.

This prospective observational study was designed to estimate spontaneous regression of biopsy-proven CIN2/3 in a short time period. In this trial, we found the overall rate of spontaneous regression of CIN2/3 to be 28%. We found that the rate of lesion regression was strongly inversely associated with infection with HPV16 and that the *HLA*A201* allele was associated with disease persistence. Should our findings be validated in a larger data set, then future interventional trials in women with CIN 2/3 may require stratification for HPV as well as HLA alleles to take into account differing rates of spontaneous regression.

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## References


1. Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med 1998;338:423–8.
2. Holowaty P, Miller AB, Rohan T, To T. Natural history of dysplasia of the uterine cervix. J Natl Cancer Inst 1999;91:252–8.
3. Melnikow J, Nuovo J, Willan AR, Chan BK, Howell LP. Natural history of cervical squamous intraepithelial lesions: a meta-analysis. Obstet Gynecol 1998;92: 727–35.
4. Nasiell K, Nasiell M, Vaclavinkova V. Behavior of moderate cervical dysplasia during long-term follow-up. Obstet Gynecol 1983;61:609–14.
5. Stoler MH, Schiffman M. Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study. JAMA 2001;285:1500–5.
6. Woodhouse SL, Stastny JF, Styer PE, Kennedy M, Praestgaard AH, Davey DD. Interobserver variability in subclassification of squamous intraepithelial lesions: results of the College of American Pathologists Interlaboratory Comparison Program in Cervicovaginal Cytology. Arch Pathol Lab Med Nov 1999;123: 1079–84.
7. Gravitt PE, Peyton CL, Apple RJ, Wheeler CM. Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. J Clin Microbiol 1998;36:3020–7.
8. Gravitt PE, Peyton CL, Alessi TQ, et al. Improved amplification of genital human papillomaviruses. J Clin Microbiol 2000;38:357–61.
9. Gravitt PE. Reproducibility of HPV16 and HPV18 viral load quantitation using Taqman real-time PCR assays. J Virol Methods 2003;112:23–33.
10. Yuan CC, Miley W, Waters D. A quantification of human cells using an ERV-3 real time PCR assay. J Virol Methods Feb 2001;91:109–17.
11. Narod SA, Thompson DW, Jain M, Wall C, Green LM, Miller AB. Dysplasia and the natural history of cervical cancer: early results of the Toronto Cohort Study. Eur J Cancer 1991;27:1411–6.
12. Follen M, Atkinson EN, Schottenfeld D, et al. A randomized clinical trial of 4-hydroxyphenylretinamide for high-grade squamous intraepithelial lesions of the cervix. Clin Cancer Res 2001;7: 3356–65.
13. Meyskens FL Jr, Surwit E, Moon TE, et al. Enhancement of regression of cervical intraepithelial neoplasia II (moderate dysplasia) with topically applied all-*trans*-retinoic acid: a randomized trial. J Natl Cancer Inst 1994;86:539–43.
14. Keefe KA, Schell MJ, Brewer C, et al. A randomized, double blind, Phase III trial using oral β-carotene supplementation for women with high-grade cervical intraepithelial neoplasia. Cancer Epidemiol Biomarkers Prev 2001;10:1029–35.
15. Moscicki AB, Hills N, Shiboski S, et al. Risks for incident human papillomavirus infection and low-grade squamous intraepithelial lesion development in young females. JAMA 2001;285: 2995–3002.
16. Kjaer S. Type specific persistence of high risk human papillomavirus (HPV) as indicator of high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study. BMJ 14 September 2002;325:572–8.
17. Dalstein V, Riethmuller D, Pretet JL, et al. Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. Int J Cancer Sep 1 2003;106: 396–403.
18. Enomoto T, Haba T, Fujita M, et al. Clonal analysis of high-grade squamous intra-epithelial lesions of the uterine cervix. Int J Cancer 1997;73: 339–44.
19. Park TW, Richart RM, Sun XW, Wright TC Jr. Association between human papillomavirus type and clonal status of cervical squamous intraepithelial lesions. J Natl Cancer Inst 1996;88:355–8.
20. Herrero R, Hildesheim A, Bratti C, et al. Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica. J Natl Cancer Inst Mar 15 2000;92:464–74.
21. Jacobs MV, Walboomers JM, Snijders PJ, et al. Distribution of 37 mucosotropic HPV types in women with cytologically normal cervical smears: the age-related patterns for high-risk and low-risk types. Int J Cancer Jul 15 2000;87:221–7.
22. Molano M, Posso H, Weiderpass E, et al. Prevalence and determinants of HPV infection among Colombian women with normal cytology. Br J Cancer Jul 29 2002;87:324–33.
23. Sherman ME, Wang SS, Wheeler CM, et al. Determinants of human papillomavirus load among women with histological cervical intraepithelial neoplasia 3: dominant impact of surrounding low-grade lesions. Cancer Epidemiol Biomarkers Prev Oct 2003;12: 1038–44.
24. Alric LFM, Izopet J, Vinel J, et al. Genes of the major histocompatibility complex class II influence the outcome of hepatitis C virus infection. Gastroenterology 1997;113:1675–81.
25. Thio CL, Thomas DL, Karacki P, et al. Comprehensive analysis of class I and class II HLA antigens and chronic hepatitis B virus infection. J Virol Nov 2003;77: 12083–7.
26. Trachtenberg E, Korber B, Sollars C, et al. Advantage of rare HLA supertype in HIV disease progression. Nat Med Jul 2003;9:928–35.
27. Sarantopoulos S, Lu L, Cantor H. Qa-1 restriction of CD8+ suppressor T cells. J Clin Invest Nov 2004; 114:1218–21.
28. Kumar V. Homeostatic control of immunity by TCR peptide-specific Tregs. J Clin Invest Nov 2004; 114:1222–6.
29. Jiang H, Chess L. An integrated view of suppressor T cell subsets in immunoregulation. J Clin Invest Nov 2004;114:1198–208.
30. Khakoo SI, Thio CL, Martin MP, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science Aug 6 2004;305: 872–4.
31. Brabin L. Interactions of the female hormonal environment, susceptibility to viral infections, and disease progression. AIDS Patient Care STDS 2002; 16:211–21.
32. Franklin RD, Kutteh WH. Characterization of immunoglobulins and cytokines in human cervical mucus: influence of exogenous and endogenous hormones. J Reprod Immunol 1999;42:93–106.
33. Ylitalo N, Sorensen P, Josefsson A, et al. Smoking and oral contraceptives as risk factors for cervical carcinoma *in situ*. Int J Cancer 1999;81:357–65.
34. Winkelstein W Jr. Smoking and cervical cancer—current status: a review. Am J Epidemiol 1990;131: 945–57; discussion 958–60.
35. Szarewski A, Jarvis MJ, Sasieni P, et al. Effect of smoking cessation on cervical lesion size. Lancet 1996;347:941–3.
36. Szarewski A, Maddox P, Royston P, et al. The effect of stopping smoking on cervical Langerhans' cells and lymphocytes. BJOG 2001;108:295–303.
37. Moreno V, Bosch FX, Munoz N, et al. Effect of oral contraceptives on risk of cervical cancer in women with human papillomavirus infection: the IARC multicentric case-control study. Lancet 2002; 359:1085–92.
38. Gram IT, Austin H, Stalsberg H. Cigarette smoking and the incidence of cervical intraepithelial neoplasia, grade III, and cancer of the cervix uteri. Am J Epidemiol 1992;135:341–6.
39. Ho GY, Kadish AS, Burk RD, et al. HPV 16 and cigarette smoking as risk factors for high-grade cervical intra-epithelial neoplasia. Int J Cancer 1998; 78:281–5.
40. Eppel W, Worda C, Frigo P, Manavi M, Czerwenka K. The influence of cotinine on interleukin 6 expression in smokers with cervical preneoplasia. Acta Obstet Gynecol Scand 2000;79:1105–11.
41. McCann MF, Irwin DE, Walton LA, Hulka BS, Morton JL, Axelrad CM. Nicotine and cotinine in the cervical mucus of smokers, passive smokers, and nonsmokers. Cancer Epidemiol Biomarkers Prev 1992;1:125–9.
42. Poppe WA, Peeters R, Daenens P, Ide PS, Van Assche FA. Tobacco smoking and the uterine cervix: cotinine in blood, urine and cervical fluid. Gynecol Obstet Invest 1995;39:110–4.
43. Prokopczyk B, Cox JE, Hoffmann D, Waggoner SE. Identification of tobacco-specific carcinogen in the cervical mucus of smokers and nonsmokers. J Natl Cancer Inst 1997;89:868–73.
44. Bell MC, Crowley-Nowick P, Bradlow HL, et al. Placebo-controlled trial of indole-3-carbinol in the treatment of CIN. Gynecol Oncol 2000;78: 123–9.
45. Follen M, Schottenfeld D. Surrogate endpoint biomarkers and their modulation in cervical chemoprevention trials. Cancer 2001;91:1758–76.