Risk Factors for Squamous Intraepithelial Lesions (SIL) of the Cervix
Among Women Residing at the US-Mexico Border

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List of Abbreviations

Statistics:
k – Kappa
U – Units
<, >, ≥, ~ – less than, greater than, greater than or equal to, approximately
n – Sample size
OR – Odds ratios

Medical and Chemical:
HPV – Human papillomavirus
ASCUS/AGUS – atypical squamous cells of undetermined significance or atypical glandular cells of undetermined significance
LSIL – Low grade squamous intraepithelial lesions
HSIL – High grade squamous intraepithelial lesions
DNA – Deoxyribonucleic acid
Pap smear – Papanicolaou smear
CIN – Cervical intraepithelial neoplasia
PCR – Polymerase chain reaction
Proteinase K – Blue juice (Digene Corp.) used as a digestion solution
Tris – Hydroxymethyl-aminomethane
AmpliTaq – (Perkin Elmer) Taq polymerase
MgCl2 – Magnesium chloride
dCTP – Deoxycytidine-5’-triphosphate
dGTP – Deoxyguanosine-5’-triphosphate
dATP – Deoxyadenosine-5’-triphosphate
dUTP – Deoxyuridine-5’-triphosphate
dTTP – Deoxithymidine-5’-triphosphate
PGMY09, PGMY11, B PC04, B GH20, PGMY09/11 L1, PGMY – HPV consensus primer system – biotinylated primers that amplify 450 base pair fragment of the L1 open reading frame of genital HPVs
Uracil N glycosylase – Incorporation of this into the PCR procedure can help to avoid PCR product carryover by digesting previously amplified material prior to subsequent amplification
GH20/PC04 – Biotinylated primers that amplify a 268 base pair fragment of the human β-globin gene
H2O – Water
CaSki – Cell line (cervical) (American Type Culture Collection, Manassas, VA)
HCII – Hybrid capture II
CIS – Carcinoma in situ
RLU – Relative light units

Measures:
ml, μl, mM, μM, M – Milliliters, microliters, millimolar, micromolar, molar
C – Celsius
rpm – Revolutions per minute

Miscellaneous:
i.e. – That is
vs. – Versus
v – Versus

Places:
MD – Maryland
TX – Texas
US – United States
ABSTRACT

It is now well established that cervical cancer is caused by oncogenic human papillomavirus (HPV) infections that commonly infect women worldwide. What remains to be understood are the factors that contribute to cervical cancer in the presence of HPV infection. We conducted a case-control analysis of women recruited at the US-Mexico border to evaluate factors associated with three cytologic outcomes simultaneously, atypical squamous cells of undetermined significance or atypical glandular cells of undetermined significance (ASCUS/AGUS), low grade squamous intraepithelial lesions (LSIL), and high grade squamous intraepithelial lesions (HSIL). A cross-sectional binational study of 2059 women ages 15-79 years was conducted between 1997-1998. A significant difference in the distribution of cytologic categories by country was observed (3.0% vs. 0.7% HSIL among Mexican vs. US women respectively). The only factors independently associated with all three cytology outcomes were HPV infection and viral load. A linear increase in risk with increasing viral load was observed for each of the three outcome variables, with the strength of this association increasing with cytology abnormality. In addition to HPV infection, parity and Mexico as a country of residence appear to be associated with LSIL and HSIL respectively. Factors associated with cytology outcomes in analyses limited to women with HPV infection were similar to results obtained in models where HPV infection was included as a covariate. Future work is needed to evaluate the predictive value of HPV viral load utilizing more specific and quantitative measures.
INTRODUCTION

It is now well established that cervical cancer is caused by oncogenic human papillomavirus (HPV) infections that commonly infect women worldwide.\textsuperscript{1} More recent reports from HPV natural history studies have demonstrated that HPV infections are predominantly transient and that only a minority of infections persist and progress to clinically relevant lesions. What remains to be understood are the factors that contribute to cervical cancer in the presence of HPV infection. Several HPV co-factors have been under investigation including smoking, parity, oral contraceptive use, nutritional status, and cervical inflammation.\textsuperscript{2,3} Depending on the study population and statistical method used to adjust for HPV infection differing results have been obtained.

To investigate the factors that contribute to cervical carcinogenesis in the presence of HPV infection most researchers have conducted case-control studies and have either statistically adjusted for HPV infection in their models or limited their analyses to HPV positive women.\textsuperscript{2} In the present study we conducted a case-control analysis of women recruited at the US-Mexico border\textsuperscript{4} to evaluate factors associated with three cytologic outcomes simultaneously, atypical squamous cells of undetermined significance or atypical glandular cells of undetermined significance (ASCUS/AGUS), low grade squamous intraepithelial lesions (LSIL), and high grade squamous intraepithelial lesions (HSIL). We present analyses to suggest that both statistical approaches to identifying HPV co-factors yield similar results in this binational population.
MATERIAL AND METHODS

Study Design

A cross-sectional survey was conducted from January 1997 to June 1998, recruiting women 15 years and older living at the Arizona, US-Sonora, Mexico border. A detailed description of the study design and methods of the US-Mexico Border Binational HPV, Cervical Dysplasia, and Chlamydia trachomatis Infection Study has been previously published. Briefly, participants were women who self-referred to Community Health Centers, County Health Departments, and Planned Parenthood clinics in Arizona, and Public Health Clinics in Sonora for routine gynecological care. Women who were (a) 15 years and older, (b) residents of Arizona-Sonora border communities, Hermosillo, Sonora, or Tucson, Arizona, (c) non-pregnant or a minimum of two months post-partum, and (d) still having menstrual periods (e.g., no hysterectomy) were invited to participate in this study. The overall response rate was 92.8% (2436 women interviewed/2626 women approached for participation). There were 2578 women who met the study eligibility criteria. Study participants (94.5% of eligible women) completed an interviewer administered risk factor questionnaire that assessed reproductive, sexual and medical histories, and demographic data. Of the 2436 women interviewed, 2319 women had a sample adequate for HPV detection and completed a questionnaire. Of these women 2059 also had complete cytology data and were included in these analyses.

After the Pap smear was obtained for routine clinical purposes, two additional samples of exfoliated cervical cells were obtained. The first of these two additional samples was utilized for the analysis of HPV DNA and the second of the two additional
samples was utilized for *Chlamydia trachomatis* determination. Specimens for *Chlamydia trachomatis* analysis were collected from the endocervical os or the cervical canal.

**Cytology**

A major focus of the study was the development of a detailed cytological coding system that was used by study pathologists in both countries to ensure the consistency of Pap smear diagnoses. Prior to initiation of this cross-sectional study we developed a common schema for reporting/recording Pap smears. Full details of this work have been previously published. The final cytological coding system that we developed for our study combined elements of both the Bethesda System and the cervical intraepithelial neoplasia (CIN) system. The diagnostic categories included specific diagnoses and evaluation of specimen adequacy. Overall, the degree of agreement between the two countries with respect to cytologic diagnoses was excellent, with a kappa value of 0.80 when four cytology categories were used (normal, ASCUS/AGUS, LSIL, and HSIL). The major areas of disagreement between raters were primarily in the diagnosis of ASCUS/AGUS and LSIL with the majority of the differences attributable to a more severe grading by the cytopathologist in Mexico compared with the US.

Cervical cytology specimens for this study were collected using the Ayre spatula to sample the ectocervix, and a cytobrush to sample the endocervix/transformation zone. Healthcare workers in each of the clinic sites received training in the proper collection technique from members of the Department of Obstetrics and Gynecology of the University of Arizona.
Pathologists in the laboratories with which the particular clinics were affiliated read all Pap smear specimens collected from the four sites in the United States initially. One study pathologist (JF) reread the cytology slides from the US in a blinded fashion, and those diagnoses were reported for the study using the system developed collaboratively. Cytologists in the Sonora state laboratory of the Secretary of Public Health initially read Pap smears from women enrolled in Mexico. Another study pathologist (EMBG) then rescreened these Pap smears, adhering to the cytology-coding schema developed for this study. The two study pathologists then reviewed all of the abnormals and 10% of the normals from both countries. The inter-rater agreement was 80.1% (κ = 0.729) for United States review of Mexican slides. The major areas of disagreement were in the diagnoses of low-grade lesions and in ASCUS/AGUS categories.\textsuperscript{4}

\textit{Sampling and Determination of HPV Status}

Cervical cells were collected for HPV analysis from the ectocervix and endocervix using the Cone Brush Cytosoft and immediately suspended in 0.6 ml Digene Diagnostics Sample Transport Medium (Digene Corporation, Gaithersburg, MD). Following collection, samples were placed and maintained at 4°C until shipment for a maximum of 2 weeks at which point they were transported on ice. Samples were then maintained at −70°C until analysis. A total of 2319 women provided cervical cell samples for HPV detection. Of these 2246 were evaluable by polymerase chain reaction (PCR). Cervical cytology results were available for 2059 of these women and comprise the final sample for this study.
HPV detection was conducted using PCR among all the cervical cell samples collected. Genomic DNA was extracted following standard techniques. In brief, 50μl aliquots were digested with 5μl Proteinase K for 1 hr at 65°C, followed by 5M Ammonium Acetate and Ethanol precipitation. The crude DNA pellet was dried and resuspended in 50μl 10mM Tris, pH 7.5. The DNA extracts were then stored at -80°C until amplification. Specimens were tested for the presence of HPV by amplifying 5μl of the DNA extracts with the PGMY09/11 L1 consensus primer system7 and AmpliTaq Gold polymerase (Perkin-Elmer, Foster City, Ca.). In brief, each amplification contained 10X PCR Buffer II, 25 mM MgCl2, 200μl (each) dCTP, dGTP, and dATP, 600μl dUTP, 5 U of AmpliTaq Gold, 50μM PGMY09, 50μM PGMY11, 50μM B_PC04, 50μM B_GH20, and 5μl of the template. For eventual inclusion of uracil-N-glycosylase to prevent product carryover, dTTP was replaced with dUTP. To determine specimen adequacy, the GH20/PC04 human β-globin target was coamplified with the HPV consensus primers. For every 10 samples a negative control (H2O) and a positive control (CaSki Cells) was run to control for contamination and accuracy. The samples were amplified using Perkin-Elmer GeneAmp PCR System 9700. The following amplification profile was used: 95°C hotstart for 9 min., 95°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 1 min for 40 cycles; followed by a 5 min terminal extension at 72°C; and a hold step at 4°C. HPV genotyping was conducted using the reverse line blot method7 on all samples that were positive by PCR. This detection method utilizes the HPV L1 consensus PCR products labeled with biotin to detect 27 HPV types. The HPV genotype strip contains 29 probe lines, detecting 27 individual HPV genotypes and two concentrations of the β globin control probe. All reagents were
kindly provided by Roche Molecular Systems Inc. (Alameda, CA). The following types were detected: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51 to 59, 66, 68, 73, 82, 83, and 84. Briefly, the PCR products labeled with biotin were denatured and added to the probe strip in a hybridization buffer. After strips were washed, streptavidin-horseradish peroxidase was added to facilitate detection of the various HPV types. After final wash, buffer was removed by vacuum aspiration, and strips were rinsed in 0.1M sodium citrate. Color development was activated by incubation in a mixture of hydrogen peroxide in sodium citrate buffer and tetramethylbenzidine in dimethylformamide for five minutes on a rotating platform (70rpm). Developed strips were interpreted and photographed for future reference. Strip interpretation was performed with a labeled overlay, with lines indicating the position of each probe relative to the reference mark.

The oncogenic HPV types associated with cervical dysplasia and cancer included 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The non-oncogenic types detected were 6, 11, 26, 40, 42, 53, 54, 55, 57, 66, 73, 82, 83, and 84.

All specimens were also analyzed by Digene Hybrid Capture II technology, utilizing Probe B, which is designed to detect oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The Hybrid Capture II technology is a signal amplified hybridization antibody capture microplate assay. It utilizes chemiluminescent detection in which the presence of HPV is detected by light emitted and then measured as relative light units (RLUs). An RLU positive control ratio of ≥ 1 is regarded as HPV-positive for one or more for the above-mentioned oncogenic HPV types. Specimens with RLU cutoff value ratios < 1 are considered "negative" or "nondetected." The sensitivity of the test is 1 picogram of viral DNA per 100 μl. To ensure that samples were accurately classified,
those samples testing with an RLU ratio of 1.0-2.0 were retested two subsequent times by Hybrid Capture. Only samples that had RLU ratios greater than 1.0 on two of the three tests were classified as HPV-positive by the Hybrid Capture method. The low-risk probe was not utilized in this study; therefore nononcogenic HPV types were not detected using the Hybrid Capture II method. Categories of viral load were determined based on the distribution of RLU values among all study participants as follows: RLU <1, 1-49, and ≥50.

There were discrepancies between HCII and PCR in our study. Using Probe B, which is designed to detect only oncogenic infections, we detected infections that were considered non-oncogenic (n=36) and were considered HPV negative (n=40) based on our PCR analyses. Estimates of viral load were calculated based on HCII results regardless of the results by PCR (e.g., HPV negative or positive for non-oncogenic infections by PCR).

Testing for Chlamydia trachomatis infection was conducted using the Digene CT-ID Hybrid Capture II (Digene Corporation, Gaithersburg, MD) test, a signal amplified hybridization microplate assay which utilizes chemiluminescence for qualitative detection of Chlamydia trachomatis DNA.4

Statistical Analysis

To investigate the relationship between predictor variables and cervical cytology we looked at the magnitude of the statistical association by calculating the age-adjusted odds ratios (OR) for each variable using polytomous logistic regression. To further evaluate which of these variables were potential confounders and factors independently
associated with cytological diagnoses ASCUS/AGUS, LSIL, and HSIL, the method of weighted least squares was used to estimate maximum-likelihood polytomous logistic models. In this multinomial logistic model women with normal cytology were the base category for the dependent variable. The model estimated three odds ratios simultaneously for each risk factor evaluated: the odds ratio for ASCUS/AGUS vs. normal, LSIL vs. normal, and HSIL vs. normal. Variables associated at p<0.20 in univariate models were considered for inclusion in final models predicting the three outcomes. Two different sets of analyses were conducted. First, all women were considered and the risk associated with HPV infection was estimated and statistically adjusted for in assessing factors independently associated with cytology outcomes. In the second set of analyses, only HPV positive women, women testing positive to the PGMY primer, were included in statistical models. Due to small numbers of women presenting with AGUS (n=19), these women were combined with women presenting with ASCUS in the final models. Restricting analyses to those with ASCUS only in this category did not significantly alter the point estimates nor change the significance of the factors associated with each of the outcomes. All analyses were conducted using Intercooled Stata/SE (StataCorp. 2001. Stata Statistical Software: Release 7.0. College Station, TX: Stata Corporation).

RESULTS

Prevalence of ASCUS/AGUS, LSIL, and HSIL and HPV infection by country (US vs. Mexico) is presented in Table 1. A significant difference in the distribution of cytologic categories by country was observed (p<0.001). The prevalence of LSIL and
HSIL was 3.0% and 2.0% among Mexican women compared with 0.7% and 0.9% among US women, respectively. In general, a higher prevalence of HPV infection was detected in US women for every cytological category considered, and a higher viral load was detected among women with oncogenic compared to non-oncogenic HPV infections. Multiple HPV infections were detected in 1.3% of normal, 5.3% of ASCUS/AGUS, 12.5% of LSIL and 10% of HSIL. Median viral load was highest among women with LSIL (1499.6 among women with single oncogenic infections) with no statistical difference when single and multiple infections were compared (p = 0.4131).

In crude analyses ASCUS/AGUS was associated with *Chlamydia trachomatis* infection, HPV infection, viral load, lifetime number of sexual partners, and number of new sexual partners in the past three months (Table 2). LSIL was associated with HPV infection, HPV viral load, concurrent *Chlamydia trachomatis* infection, marital status, country of clinic, having a Pap smear in the past three years, lifetime number of male partners, number of new partners in the past three months, parity, and current oral contraceptive use. HSIL was associated with country of clinic, HPV infection, viral load, having a Pap smear in the past three years, and current use of injectable contraceptives.

Factors independently associated with ASCUS/AGUS, LSIL, and HSIL among all study participants and limited to HPV positive women are presented in Table 3. The only factors associated with all three cytology outcomes among all study participants were HPV infection and viral load. The strength of the association with oncogenic HPV infection increased linearly with increasing grades of abnormality (i.e., OR=3.67 for ASCUS/AGUS, 13.91 for LSIL, and 23.38 for HSIL as measured by PCR for single oncogenic infections). A linear increase in risk with increasing viral load was observed.
for each of the three outcome variables, with the strength of this association increasing with cytology abnormality (OR=5.96 for ASCUS/AGUS, 32.56 for LSIL, and 64.08 for HSIL when comparing RLU ratio of 50-2800 to <1). Other than HPV infection, ASCUS/AGUS was independently associated with concurrent Chlamydia infection (OR=2.18 for positive vs. negative women) and LSIL was associated with country (OR=3.86 for women in Mexico compared to the US) and parity (OR 4.5-11.35 for ≥1 live births compared with none). Current OC use was marginally associated with reduced risk of LSIL (OR=0.35). In addition to HPV infection, HSIL was associated with country (OR=2.66 for women in Mexico compared to the US) and marginally associated with parity (OR=2.03 for 3-4 live births compared with none) and current injectable contraceptive use (OR=2.77).

Factors associated with cytology outcomes in analyses limited to women with HPV infection (Table 3) were similar to results obtained in models where HPV infection was included as a covariate. In models restricted to HPV positive women, no non-HPV factors were significantly associated with ASCUS/AGUS, LSIL was associated with country (OR=3.08 for women in Mexico compared with the US) and parity (OR 4.58-14.23 for ≥1 live births). Country (OR=2.49 for women in Mexico compared to the US) was marginally associated with HSIL.

**DISCUSSION**

In this study of women residing at the US-Mexico border the only factor associated with all of the cytologic outcomes of ASCUS/AGUS, LSIL, and HSIL were infection with oncogenic HPV types, primarily HPV 16, and high viral load. In addition
to HPV, ASCUS/AGUS was inconsistently associated with concurrent Chlamydia trachomatis infection, LSIL was consistently associated with country of clinic and parity, and HSIL was inconsistently associated with country of clinic.

There has been recent interest in the association between HPV viral load and cervical disease. Three different methods have been utilized to estimate viral load. In older publications viral load was dichotomized as either low or high based on the combined results of PCR and hybridization methods.\textsuperscript{8-12} The second method measures the ratio of RLU's of participant samples relative to a known positive control using Digene's Hybrid Capture method.\textsuperscript{13-23} Recently, a more precise estimate of viral load has been derived from methods employing quantitative PCR.\textsuperscript{24-28} Of the cross-sectional or case-control studies published,\textsuperscript{8, 10-19, 21, 22, 24-28} most found significant associations between HPV viral load and cervical disease.\textsuperscript{8, 10, 11, 13-19, 21, 22, 24-28} The majority of studies that utilized Hybrid Capture testing methodology to estimate viral load observed an association with disease outcome. In this present study we estimated viral load from the RLU ratio using the HCII assay and we observed a linear association with risk for all cytologic categories examined. Our results from a binational US-Mexico population add to the growing consistent finding that HPV viral load is associated with cervical disease status when both are measured at the same time point.

An important question unanswered by cross-sectional and case-control studies, as well our study, is whether HPV viral load is predictive of subsequent development of cervical disease. To date only four studies have evaluated the association between HPV viral load and cervical disease prospectively.\textsuperscript{9, 20, 23, 26} The two studies that demonstrated an association specifically evaluated either lesion persistence\textsuperscript{9} or lesion progression\textsuperscript{26} as
the endpoints. Among the studies that evaluated incident disease viral load was not predictive of a HSIL\textsuperscript{20} or development of a CIN III lesion\textsuperscript{22}. It remains to be determined whether there is predictive value of viral load measures as more accurate estimates of viral load, such as those utilizing real time PCR, are assessed in prospective studies. If viral load is associated with lesion development, it will be important to understand the factors that contribute to high HPV viral load.

In this study parity was consistently associated with LSIL, but not with HSIL, adding to the literature which suggests a different role for parity at different points in carcinogenesis. In general when CIS or cervical cancer is the endpoint or comprises the case group, consistent associations with OC use and parity are observed across studies.\textsuperscript{2} Among studies of lower grade lesions parity is inconsistently associated with disease.\textsuperscript{2} In the present study we did not detect a significant association between either OC use or parity and HSIL, regardless of whether analyses were limited to HPV positive participants or HPV was adjusted for statistically in the model. However, we did detect a significant increase in risk for LSIL with increasing parity and a non-significant increase in risk among women with $\geq 3$ live births (OR$\sim2.0$) for HSIL. Unfortunately, in the present study the small number of HSIL cases (n=30) may have limited our ability to detect an association. OC use and use of other hormonally based contraceptives in this study were not associated with either LSIL or HSIL despite a relatively high prevalence of past and current OC use. Unlike previously published studies we detected no association between tobacco use and any of the cytology endpoints examined.

Receiving gynecological care in clinics in Mexico compared with the US was consistently associated with higher risk of LSIL and inconsistently associated with risk of
HSIL. This association was independent of parity, cytology screening history, concurrent *Chlamydia trachomatis* and HPV infection. Several factors may explain this observation, including differences in HPV viral load, prevalence of non-European variants of HPV 16, and prevalence of concurrent inflammation between the US and Mexico. When examined, the distribution of viral load differed between the US and Mexico, suggesting that differences in viral load may explain the increased risk of SIL. The factors that contribute to viral load need to be further evaluated in future studies. Several publications have now demonstrated significantly elevated risk of disease with non-European compared with European variants of HPV 16.\(^{29}\) Although the overall HPV 16 prevalence is similar between countries\(^ {30}\) it is possible that the prevalence of non-European variants of HPV 16 is higher in Mexico than in the US. Recent literature also suggests cervical inflammation has a pro-carcinogenic role in HPV disease.\(^ {31}, \) As we have previously shown that the prevalence of inflammatory cells was significantly higher in women who reside in Mexico compared with the US it is possible that the country effect observed here is due to differences in inflammation by country.\(^ {4}\) A higher prevalence of inflammation in Mexico may have resulted in more difficult interpretation of the Pap smear leading to a higher degree of false positives among the Mexican smears. Although not known, cervical inflammation may also be contributing to the higher viral loads observed among Mexican women in this study. Unfortunately, we do not have data on prevalence of HPV 16 variants or presence of inflammatory cells in all women who participated in this study.

As with all cross-sectional studies caution must be taken in interpreting risk factors for cervical disease, as the temporality of these associations cannot be inferred. In
addition, biopsies and histology were not conducted. However, a panel of cytopathologists was utilized for reviewing and classifying cervical cytology smears. As a consequence of not having histological results we were unable to distinguish between CIN II and III lesions. Results presented in this manuscript represent participation from women self-referring for Pap smears in each country. As such, these women may have a different overall prevalence of HPV and risk profile for cervical abnormalities compared with the underlying population in each country. In addition, only women from northern Mexico and southern Arizona were included in this study, thereby limiting the generalizability of these findings to other populations in each respective country. Our sample size to estimate associations with HSIL was small limiting us to the detection of only strong associations. Finally, a study with a larger sample size is needed to disentangle the separate and combined effects of viral load and multiple HPV infections on risk for SIL.

In conclusion, oncogenic HPV infection and high viral load were consistently associated with cervical lesion severity regardless of statistical analytical approach. In addition to HPV infection, pantry and Mexico as a country of residence appear to be associated with LSIL and HSIL respectively. Future work is needed to evaluate the predictive value of HPV viral load utilizing more specific and quantitative measures. Despite a similar prevalence of oncogenic HPV infections among women in Mexico and the US, an elevated risk of LSIL and HSIL was observed among women residing in Mexico. Only large prospective studies which incorporate careful assessment of inflammatory status, HPV variant analyses, and precise estimates of viral load can determine whether these factors are associated with cervical lesion development.
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