Multiple Human Papilloma Virus Infections and Their Impact on the Development of High-Risk Cervical Lesions

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Key Words

Cervical cancer · Cervical dysplasia · Human papillomavirus · Intergenotypic interaction · Multiple human papillomavirus infections

Abstract

Objective: Individuals are often infected with multiple genotypes of human papillomavirus (HPV) simultaneously, but the role these infections play in the development of cervical disease is not well established. This study aimed to determine the association of multiple HPV infections with high-risk cervical lesions (hrCLs). **Study Design:** HPV genotyping was performed on 798 SurePath specimens collected between December 1, 2009, and April 30, 2011. The cases were classified as hrCL (n = 90) or non-hrCL (n = 708) based on cytology diagnoses. The association between hrCL and HPV infection patterns was analyzed. **Results:** Multiple HPV infections were frequently encountered (38.2%) in the cohort. Increased frequency of hrCLs was associated with a single high-risk HPV (hrHPV) infection. An additive or synergistic effect was not observed for hrCL in multiple HPV infections. The hrCL rates appeared to decrease in various patterns of multiple HPV infections, but the reduction was not statistically significant. **Conclusions:** Multiple HPV infections are

common with no additive or synergistic effect on the development of hrCL. Conversely, reduced hrCL rates were observed in various patterns of multiple HPV infections compared to their single-genotype infection counterparts, suggestive of possible intergenotypic competition or more effective immune response triggered by multiple infections. Further studies in larger cohorts are needed.

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Introduction

Human papillomaviruses (HPVs) are small, nonenveloped, double-stranded DNA viruses, and more than 40 genotypes have been associated with infection of the female genital tract. Numerous studies have focused on infections due to various high-risk HPV (hrHPV) genotypes and their effect on the development of cervical dysplasia and cancers [1]. Based on the oncogenic potential of individual HPV genotypes, the expert working group of the International Agency for Research on Cancer (IARC) classified HPV into four groups: carcinogenic (group 1), probably carcinogenic (group 2A), possibly carcinogenic (group 2B) and not classifiable (group 3) [2].

Results from various study populations have shown that multiple HPV genotype infections are frequently encountered, and cervical infections are not always caused by a single HPV genotype [3–12]. Despite mounting reports on the outcomes of infection with an individual hrHPV genotype, the interactions between co-infecting HPV genotypes are not well studied, and the effect of multiple HPV infections on cervical disease is controversial. Several studies have demonstrated that multiple HPV infections are a significant risk factor for high-risk cervical lesions (hrCLs) [13–19]. These reports also concluded that HPV infections are linearly correlated with the severity of cervical intraepithelial neoplasia, and that the infections appear to act synergistically in cervical carcinogenesis. Conversely, alternate studies showed that multiple HPV infections have no synergistic or additive effect on the development of cervical diseases or increased risk for hrCL as compared to a single HPV infection [9, 20–24]. The findings from a more recent study, however, suggest that competitive and/or cooperative interactions may exist among certain HPV genotypes [10]. In this context, Sobota et al. [25] showed that co-infection with HPV genotypes from the same taxonomic species reduced the likelihood of progression to hrCL in HIV-positive women; co-infection with HPV genotypes from different species, however, did not confer protection from hrCL.

In view of the contradicting data reported to date, additional studies are warranted to gain mechanistic insight and elucidate the effect of multiple HPV infections on cervical dysplasia and cancer. In this study, we analyzed the HPV infection patterns in an underserved population and their association with hrCLs. The aim of the study was to determine the effect of multiple HPV infections on cervical dysplasia and cancer, and to explore the possible interactions among the HPV genotypes in women with multiple HPV infections.

Materials and Methods

Study Population and Cytology Classification

The study was conducted with approval from the Institutional Review Board of the Houston Methodist Research Institute (IRB1210-0221, approved February 25, 2011). The study included 798 women who were referred to the Houston Methodist Hospital from charity clinics in the Houston area for abnormal Papanicolaou tests from December 1, 2009, to April 30, 2011. The study population was comprised of predominantly Hispanic women who had no medical insurance and did not qualify for Medicare or Medicaid programs at the time of the study.

Liquid-based Papanicolaou tests (SurePath; BD, Franklin Lakes, N.J., USA) were performed for cytological evaluation, and

the findings were interpreted according to the criteria set by the 2001 Bethesda System for cervicovaginal cytology reporting. All abnormal Papanicolaou tests were interpreted by board-certified cytopathologists and gynecologic pathologists. Based on cytological diagnoses, the cases were divided into two general groups: those with hrCLs (90 cases), including atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H), low-grade squamous intraepithelial lesion with atypical cells, cannot exclude high-grade squamous intraepithelial lesion (LSIL-H) and high-grade squamous intraepithelial lesion (HSIL), and those with non-hrCL (708 cases), including atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL), and negative for intraepithelial lesion or malignancy (NILM).

HPV Genotyping by DNA Microarray and DNA Sequencing

HPV DNA was extracted from the residual SurePath specimens and amplified by polymerase chain reaction in the L1 region of the HPV genome before being labeled with Cy5 and hybridized with an HPV DNA microarray chip with 40 HPV genotypes (GG HPV DNA genotyping chip kit; GoodGene Inc., Seoul, Korea). The signal was visualized using a GenePix 4000B microarray scanner (Molecular Devices Inc., Sunnyvale, Calif., USA). The detection limit of the HPV 40 DNA microarray chip is between 10 and 100 copies of HPV DNA per sample. The microarray data were confirmed by direct DNA sequencing. The data obtained by automated DNA sequencing were analyzed for HPV genotypes using BLAST (Basic Local Alignment Search Tool) search (<http://www.ncbi.nlm.nih.gov/BLAST/>). DNA sequencing identified the most dominant genotype in a given specimen and served as a confirmatory assay.

Data Analysis

The HPV genotypes in IARC groups 1 and 2A are commonly referred to as hrHPV, and include genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68A/68B. In this study, IARC groups 2B and 3 are collectively referred to as non-hrHPV. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated, and Fisher's exact test was used to determine the significance of the differences in hrCL rates among the various HPV infection patterns. All analyses were performed with STATA version 13 (StataCorp LP, College Station, Tex., USA). Statistical significance was defined as two-tailed $p < 0.05$ for all tests.

Results

The average age of the patients was 36.5 years (range 19–85 years). The majority of the patients were between 20 and 39 years old ($n = 539$; 68%), which was also the dominant age group for abnormal cytology diagnoses. In the cytological diagnosis groups, hrHPV genotypes were found in 67% of women with hrCL and 38% of those with non-hrCL.

The prevalence of hrHPV and non-hrHPV among the cytological diagnosis groups is shown in figure 1. A higher proportion of hrCL was seen in women infected

Fig. 1. HPV genotypic prevalence in cytology diagnosis categories.

with hrHPV genotypes such as HPV 16, 31 and 45. The HPV infection status (single, multiple or negative) among age groups is shown in figure 2. Multiple HPV infections were observed in 38.2% of the women, whereas 54.9% of the women had a single HPV infection; no HPV was detected in 7% of the cases. The frequency of multiple HPV infection reached a maximum in the age range from 20 to 29 years and gradually decreased thereafter.

The comparison of HPV infection patterns (single or multiple) between cytology groups of hrCL and non-hrCL is shown in table 1. When all HPV genotypes were considered, the rates of hrCL were the same in women with a single or multiple HPV infections.

Table 2 summarizes the cytology results and statistics among various HPV infection patterns. In women with a single HPV infection, the hrCL rate was strongly associated with a single genotype infection of hrHPV as compared to those infected with a single non-hrHPV genotype (OR = 7.28, 95% CI: 2.99–21.27, $p < 0.0001$) or those in the HPV-negative group (OR = 6.16, 95% CI: 1.45–26.19, $p = 0.004$). In women infected with multiple HPV genotypes, hrCL was significantly associated with multiple genotypes of hrHPV compared to those infected

Fig. 2. HPV infection patterns among age groups.

Table 1. Single and multiple HPV infections in cytology groups

Cytology groups	HPV negative	Single HPV	Multiple HPV
Non-hrCL	53 (96%)	386 (88%)	269 (88%)
hrCL	2 (4%)	52 (12%)	36 (12%)

hrCL = ASC-H, LSIL-H and HSIL.

Table 2. Patterns of multiple HPV infections in cytology groups

Cytology group	HPV negative	Single HPV		Multiple HPV		
		hrHPV	non-hrHPV	hrHPV	non-hrHPV	hr + non-hrHPV
hrCL	2 (4%)	46 (19%)	6 (3%)	14 (16%)	1 (3%)	21 (12%)
Non-hrCL	53 (96%)	198 (81%)	188 (97%)	73 (84%)	38 (97%)	158 (88%)
OR (95% CI)		6.16 (1.45–26.19)	0.85 (0.17–4.31)	5.08 (1.11–23.31)	0.7 (0.06–7.97)	3.52 (0.80–15.52)
p value		0.004	1	0.03	1	0.12

hrCL = ASC-H, LSIL-H and HSIL. ORs (95% CIs) were calculated with HPV negative as the reference group.

Table 3. Number of co-infecting hrHPV genotypes in cytology groups

Cytology group	HPV negative	1 hrHPV	2 hrHPV	3+ hrHPV	Non-hrHPV
hrCL	2 (4%)	46 (19%)	11 (18%)	3 (11%)	7 (3%)
Non-hrCL	53 (96%)	198 (81%)	49 (82%)	24 (89%)	226 (97%)
OR (95% CI)		6.16 (1.45–26.19)	5.95 (1.26–28.19)	3.31 (0.52–21.13)	0.82 (0.17–4.06)
p value		0.004	0.02	0.33	0.68

hrCL = ASC-H, LSIL-H and HSIL. ORs (95% CIs) were calculated with HPV negative as the reference group.

Table 4. Infection patterns associated with HPV 16/18 and non-16/18 genotypes

Cytology group	HPV negative	HPV 16/18	Single non-16/18 hrHPV	HPV 16/18 + non-16/18 hrHPV	HPV 16/18 + non-hrHPV
hrCL	2 (4%)	23 (20%)	24 (17%)	13 (13%)	2 (6%)
Non-hrCL	53 (96%)	92 (80%)	114 (83%)	89 (87%)	29 (94%)
OR (95% CI)		6.63 (1.5–29.22)	5.58 (1.27–24.48)	3.87 (0.84–17.82)	1.83 (0.24–13.66)
p value		0.005	0.01	0.09	0.62

hrCL = ASC-H, LSIL-H and HSIL; HPV 16/18 = HPV 16 and/or 18. ORs (95% CIs) were calculated with HPV negative as the reference group.

with multiple non-hrHPV genotypes (OR = 7.29, CI: 1.02–316.06, $p = 0.04$) or those in the HPV-negative group (OR = 5.08, 95% CI: 1.11–23.31, $p = 0.03$). There were no statistically significant differences in hrCL rates when comparing other HPV infection patterns, including 1 versus 2 hrHPV genotypes, 1 versus 2 non-hrHPV genotypes, or non-hrHPV versus HPV negative. However, in women infected with combined hrHPV and non-hrHPV genotypes, the hrCL rate was somewhat reduced and more similar to those infected with non-hrHPV genotypes (OR = 5.05, 95% CI: 0.66–38.73, $p = 0.14$) or to those that were HPV negative (OR = 3.52, 95% CI: 0.80–15.52, $p = 0.12$). The decreased hrCL rate in women with

combined hrHPV and non-hrHPV infections was nevertheless not significant as compared to those with single or multiple hrHPV infections ($p = 0.06$ and $p = 0.34$, respectively).

When only hrHPV infections were considered, the women infected with 1 or 2 hrHPV genotypes had a significantly higher risk of developing hrCL with respect to the HPV-negative group ($p = 0.004$ and $p = 0.02$, respectively; table 3; fig. 3). However, the risk for hrCL in women infected with 3+ hrHPV genotypes decreased and showed no significant difference from those infected with multiple non-hrHPVs ($p = 0.68$) or those who tested negative for HPV ($p = 0.33$). The OR of hrCL among women

Fig. 3. Association of hrCL with number of hrHPV genotypes.

Fig. 4. Association of hrCL with HPV infection patterns related to HPV 16/18.

Fig. 5. ORs for HPV infection patterns associated with hrCL.

infected with 1, 2 or 3+ hrHPV genotypes was statistically insignificant ($p = 1.0$ for 1 vs. 2 hrHPV, $p = 0.43$ for 1 vs. 3+ hrHPV and $p = 0.53$ for 2 vs. 3 hrHPV). Despite being statistically insignificant, however, the risk for hrCL decreased with increasing number of co-infecting genotypes, especially when the infections involved 3+ hrHPV genotypes (fig. 3).

In light of the well-recognized role of HPV 16 and 18 in cervical dysplasia and cancer, we further analyzed the risk for hrCL in various infection patterns related to HPV 16 and 18 (table 4; fig. 4). Our analysis showed that hrCL was significantly associated with infection of HPV 16/18 (20%, $p = 0.005$) and non-16/18 hrHPV genotypes (17%, $p = 0.01$) as compared to the HPV-negative group (4%). The rates of hrCL decreased in women who had HPV 16/18 infection combined with either non-16/18

hrHPV (13%) or non-hrHPV (6%) genotypes, with no significant difference with respect to that of the HPV-negative group ($p = 0.09$ and $p = 0.62$, respectively). However, despite the downward trend observed in figure 4, the reduced hrCL rates in women with HPV 16/18 combined with non-16/18 hrHPV or non-hrHPV were not statistically significant compared to other infection patterns (table 4). The ORs (95% CI) of various HPV infection patterns associated with hrCL are shown in figure 5.

The rates of hrCL among women infected with 1, 2 or 3+ HPV species are summarized in table 5. Women infected with 3+ HPV species appeared to have a reduced rate of hrCL (7%) compared to infections with 1 (11%) or 2 species (15%). However, the differences were not statistically significant ($p = 0.26$ and $p = 0.08$, respectively).

Table 5. Number of HPV species in cytology groups

Cytology group	HPV negative	1 species	2 species	3+ species
hrCL	2 (4%)	54 (11%)	28 (15%)	6 (7%)
Non-hrCL	53 (96%)	422 (89%)	153 (85%)	80 (93%)
OR (95% CI)		3.39 (0.80–14.31)	4.85 (1.12–21.05)	1.99 (0.39–10.22)
p value		0.1	0.02	0.48

hrCL = ASC-H, LSIL-H and HSIL. ORs (95% CIs) were calculated with HPV negative as the reference group.

Discussion

In previous studies, multiple HPV infections were identified in 20–30% of the study cohorts [3–12, 20, 26–30]. In the present study of underserved Hispanic women, infection with 2+ genotypes was observed in 38.2% of the group. Women infected with hrHPV exhibited higher rates of hrCL than those infected with non-hrHPV regardless of single or multiple infections (fig. 4).

It is not clear in women with multiple HPV infections whether a competitive or cooperative interaction exists among the co-infecting genotypes. Several prior studies suggest a synergistic effect of multiple infections by HPV genotypes on oncogenesis [13–19], a conclusion that contradicts the findings of other investigators [9, 16, 20–24]. In the current study, we observed that single hrHPV infection carried the highest risk for hrCL. However, when women were co-infected with additional HPV genotypes, regardless of hrHPV or non-hrHPV, the hrCL rates were not higher as compared to the rates of the corresponding genotypes in single genotype infection or the projected hrCL rate of the combined genotypes. The results were consistent among various combinations of HPV infection patterns including hrHPV with another hrHPV, hrHPV with non-hrHPV, HPV 16/18 with non-16/18 hrHPV, or HPV 16/18 with non-hrHPV genotypes. Therefore, no additive or synergistic effect was identified in any of the various combinations of HPV infection patterns in our cohort. Conversely, we observed that multiple HPV infections were actually associated with lower rates of hrCL compared to the corresponding single HPV infection. For example, the hrCL rate decreased from 18.9 to 18.5% in women infected with 1 or 2 hrHPV genotypes, respectively, and to 11% in those with 3+ hrHPV genotypes (fig. 3). Reduced rates of hrCL were also observed in women with mixed infection of hrHPV with non-hrHPV (11.7%; table 2), HPV 16/18 with non-16/18 hrHPV (12.7%) or HPV 16/18 with non-hrHPV genotypes (6.5%; fig. 4). In addition,

infection with 3+ HPV species showed a reduced rate of hrCL (6.9%) compared to infections with 1 or 2 species (11.5 and 15.4%, respectively; table 5). The odds of having hrCL decreased in the women co-infected with additional genotypes as compared to those infected with a single genotype of HPV 16 (OR = 10.70); the decrease was 38% with non-16/18 hrHPV (OR = 6.63), 59% with HPV 18 (OR = 4.42), 68% with HPV 18 and non-16/18 hrHPV (OR = 3.46) and 77% with non-hrHPV (OR = 2.41; fig. 5). Although the differences were not statistically significant in this relatively small cohort, the observed trend suggests the possibility of competitive interactions among certain HPV genotypes in individuals with multiple HPV infections. The aforementioned findings are in agreement with a recent study showing cross protection from progression to cervical cancer if co-infection with HPV types from the same species has occurred [25]. Our results are also in accordance with the established finding that women who are seropositive for both HPV 16 and HPV 6 are less likely to develop cervical cancer as compared to those who are seropositive only for HPV 16, a mechanism known as viral antagonism [31, 32]. Antagonistic interactions were also detected for combinations of HPV 16 and HPV 18 as well as HPV 16 and HPV 33 [31]. However, the mechanisms of the possible intergenotypic competition are likely more complex and may involve multiple key stages of the HPV infection process, including binding sites/receptors, utilization of host cell organelles, synthesis of viral DNA, insertion of viral DNA into the host genome and expression of viral proteins. A better understanding of the interactions among HPV genotypes is of great interest, not only for risk assessment of hrCLs in women with multiple HPV infections, but also for the development of the next generation of HPV vaccines by predicting the possible emerging genotypes that compete with those targeted by current vaccines (i.e. HPV 16 and 18). Further studies including larger cohorts are needed to elucidate the underlying mechanisms.

In addition to the possible competitive effect among HPV genotypes, the host immune response should also be considered when evaluating the reduced risk for hrCL in women with multiple HPV infections. It has been shown that cervical HPV infection is higher in populations with impaired immune responses, such as HIV infection [33, 34] and cigarette smoking [35–37]. Natural HPV infections usually produce ineffective neutralizing antibodies in less than half of the infected women. In women with multiple HPV infections, the concurrent infection of multiple genotypes may collectively induce a more effective local or humoral immune response than that triggered by a single HPV infection. Alternatively, the weak and ineffective immunity generated by each HPV genotype may collectively provide an overall stronger immunity against HPV infection through antibody cross-reactions [36]. In support of this, cross-protective vaccine efficacy against infections and lesions associated with HPV 31, 33, and 45 was reported for both the bivalent and quadrivalent HPV vaccines [38]. The cross immunoreactions may effectively reduce or prevent viral invasion and replication, thus decreasing the risk for developing hrCLs.

Despite the observed trends in our present investigation, our results need to be interpreted cautiously as the study cohort is a high-risk population with a high HPV infection rate and it exhibits a different genotypic pattern from the general screening population in the United States [39]. In this respect, the findings from this study may not be applicable to other populations. In addition, this was a cross-sectional study without follow-up data evaluating the long-term effects of multiple HPV infections. Lastly, in this study we aimed to investigate the association between hrCL and various HPV infection patterns, without taking into account other factors such as

HIV status, tobacco use or sexual history, which may also be risk factors for hrCLs.

In conclusion, hrHPV infection was strongly associated with hrCL compared to infection with non-hrHPV regardless of single or multiple infections. Multiple HPV infections occurred frequently within the cohort, but they did not appear to carry an increased risk for hrCL compared to the women infected with the corresponding single HPV genotype. An additive or synergistic effect for hrCL was not identified in women infected with multiple HPV genotypes. A possibly reduced risk for hrCL was observed in women infected with various combinations of multiple HPV compared to their single-genotype-infected counterparts, although the differences were not statistically significant. The reasons underlying these findings are not yet known, but possible intergenotype competition or the mounting of a more effective immune response could be contributing factors. Additional studies in larger, more diverse populations are warranted to elucidate the role of multiple HPV infections in cervical dysplasia and cancer.

Acknowledgments

The authors thank Woo-Chul Moon, MD, PhD, for sponsoring the HPV genotyping tests, and Helen Chifotides, PhD, and Kathryn E. Stockbauer, PhD, for editing of the paper. This study was not supported by any specific funding source.

Disclosure Statement

The authors of this study have no relevant financial interests to report.

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