Original Article
High Grade Cervical Intraepithelial Neoplasia and Viral Load of High-Risk Human Papillomavirus: Significant Correlations in Patients of 22 Years Old or Younger

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Abstract: High-risk human papillomavirus (HR-HPV) is recognized as the primary cause for the development of cervical cancers and their precursor lesions. We investigated whether high-grade cervical dysplasia correlates with high viral load of HR-HPV in an age-dependent manner. Cases were retrospectively selected to include patients with a prior cytological diagnosis of ASCUS or higher grade squamous intraepithelial lesions, and a positive Digene Hybrid Capture II (HC-II) HR-HPV testing within 2 months before or after cervical biopsy. The quantitative viral load data was classified as negative, low, moderate and high according to the manufacturer's instruction. Cases were then stratified into 4 age groups: ≤22 years, 23-30 years, 31-40 years and >40 years. Chi-Square analysis and logistic regression were performed where appropriate. A total of 995 patients were identified. Age categories were significantly associated with HPV loads (p=0.046). Moderate to high viral loads of HPV were significantly related to the histological grade of dysplasia (p=0.029). Logistic regression analysis further confirmed the association of HPV with histological grades, even after adjusting for age factor. In particular, high-grade dysplasia (p=0.011) but not low grade dysplasia (p=0.140) was significantly associated with moderate to high HPV loads. Patients of 22 years old or younger were the only group found significantly correlated with high viral loads of HPV (p=0.015). In conclusion, high-grade squamous intraepithelial lesions and patients' age of 22 years old or younger are significantly associated with a moderate to high viral load of HR-HPV.

Key Words: High-risk human papillomavirus (HR-HPV), age, viral load, cervical intraepithelial neoplasia (CIN)

Introduction

Clinical and epidemiological studies have confirmed that high-risk human papillomavirus (HR-HPV) infection (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 59, and 68) is the direct cause of cervical intraepithelial neoplasia (CIN) and cancers [1, 2]. However, the relationship between HR-HPV viral load and histological severity of cervical precancerous lesions is still controversial. Some studies have found that different levels of HR-HPV correlate with grades of CIN [2, 3, 4, 5, 6], while others have reported no significant

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correlations [7, 8, 9], or only a high viral load of HPV-16 positively associated with the severity of cervical lesions [1, 10, 11, 12, 13]. Other studies revealed lower amounts of HR-HPV in the cervical samples of women with CIN3 compared with CIN2 [14]. Some authors reported a progressive decline of HR-HPV positivity as age increases and prevalence of HR-HPV infection strongly associated with young age [8]. One of the remaining questions is whether a high viral load of HR-HPV has an age-dependent relationship with high-grade cervical dysplasia. In this study, we investigated the relationship between the viral load of HR-HPV infection and the degree of CIN in an age-dependent manner, paying special attention to patients with ages between 15

and 22 years.

Material and Methods

Study Population

The study consisted of a retrospective screening of estimated 4,000 patients who underwent colposcopic cervical biopsies or loop electrosurgical excision procedure (LEEP) accessioned at Yale New Haven Hospital (YNHH) from August 1st 2005 to July 30th 2006. All patients had a prior cytological diagnosis of atypical squamous cells of unknown significance (ASCUS) or higher grade squamous intraepithelial lesions. Final selection of cases included only patients who had both a cervical smear for cytological diagnosis and a positive HR-HPV testing within 2 months before or after a cervical biopsy. Criteria for exclusion included: women who had either a cervical smear or HPV test but not both, an interval between the cervical smear and the biopsy was over 2 months, detection of invasive cervical carcinoma, inadequate smear, or liquid-based preparation cytology samples that could not be used for HPV testing.

Cytological Diagnosis

All patients had a gynecologic examination at YNHH. When conventional cytology was performed, a cytological smear was taken using an Ayres spatula, and then a sample for the HC-II test was kept and sent when appropriate. These samples were suspended in 1 ml of STM transport medium and stored at −20°C until further processing. If the liquid-based cytology was chosen, only one cervical scrape with a Cervix brush was utilized. Samples were prepared in PreservCyt medium for liquid-based cytology using the ThinPrep

technique (Cytyc Corporation). Four mililiters of the sample were centrifuged. Subsequently, the cell pellet was resuspended in 200 µl of phosphate-buffered saline for HPV testing. Smears were classified according to the Bethesda system for reporting cervical cytological diagnosis. Cytotechnicians and cytopathologists involved in the study were not informed about the results of the HPV testing. A committee of staff members including cytopathologists reviewed discrepant cases monthly reaching a final diagnosis.

HPV Testing

HPV DNA testing was performed by the Hybrid Capture II (HC-II) system according to the manufacturer's instructions (Digene), using the specific HPV RNA probe cocktail for carcinogenic HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Details on the conduct and analysis of cytology and testing for HR-HPVs by the Hybrid Capture Assay have been described previously [14]. Presence or absence of HPV DNA in the specimen was defined according to strength in relative light units (RLU). HPV viral load in RLU to positive controls (RLU/PC) were categorized for analysis into three groups: low viral load (1 to 10), moderate viral load (10 to 100), and high viral load (>100).

Histological Diagnosis

All women included in the study had a colposcopy within 2 months before or after the HPV testing. Punch biopsies were taken from the areas colposcopically suspicious for CIN, and endocervical curettages were performed when indicated. Some patients had cervical cone or LEEP when recent biopsy showed high grade CIN (CIN 2 or 3) or unsuccessful colposcopy. The diagnosis of cervical

Table 1 Relationship between different age groups and cytological diagnosis

Age	# Cases	Cytological Diagnosis			P value
		# ASCUS (%)	# LSIL (%)	# HSIL (%)	
≤22	256	145 (56.6)	105 (2.34)	6 (41.0)	0.823
23-30	312	193 (61.9)	109 (3.21)	10 (34.9)	
31-40	212	131 (61.8)	76 (2.36)	5 (35.9)	
>40	215	127 (59.1)	82 (2.79)	6 (38.1)	
≤22	256	451 (56.6)	267 (2.34)	21 (41.0)	0.368
>22	739	145 (61.0)	105 (2.84)	6 (36.1)	

ASCUS: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion
HSIL: high-grade squamous intraepithelial lesion

Table 2 Relationship between different age groups and histological diagnosis

Age	# Total cases (%)	Histological Diagnosis		
		# ND (%)	# HPV+CIN (%)	# CIN2+3 (%)
≤22	256 (25.7)	31 (12.1)	170 (66.4)	55 (21.5)
23-30	312 (31.4)	44 (14.1)	207 (66.4)	61 (19.6)
31-40	212 (21.3)	33 (15.6)	128 (60.4)	51 (24.1)
>40	215 (21.6)	24 (11.2)	159 (74.0)	32 (14.9)

ND: negative for dysplasia; HPV+CIN1: HPV infection and cervical intraepithelial neoplasia 1; CIN2+CIN3: cervical intraepithelial neoplasia 2 and 3

squamous lesions was based on well-established criteria into no dysplasia, HPV cytopathic changes, CIN1, CIN2 and CIN3.

Statistical Analysis

The Chi-Square test was employed to examine the relationship of histology with HPV as well as age categories with HPV. Age was sorted into four groups: 22 or younger, 23-30, 31-40, and over 40. Histology was classified as 'no dysplasia', 'low' (HPV cytopathic changes and CIN1) and 'high' (CIN 2 and 3). To further investigate the nature of these relationships, we dummy coded each category and analyzed these variables in a logistic model. SAS software was used in data coding and data analysis. An alpha level of 0.05 was set for significance.

Results

A total of 995 patients fulfilling the selection criteria were eventually identified. The median age was 31.5 years (ranging 15 to 79 years). **Table 1** shows the relationship between the patients' age and the severity of cytological diagnosis. The numbers of patients in each age group were comparable, ranging from 212to 312 cases. Percentages of high grade dysplasia (HSIL) were similar among all age groups, ranging from 34.9% to 41.0% and no statistically significant association was observed (p=0.823), even after combining the patients into two age groups, ≤22 and >22

years old (p=0.368).

The histological diagnosis based on age groups is given in **Table 2**. High-grade dysplasias (CIN 2+3) were relatively uncommon, representing 14.9 to 24.1% among age groups, and low-grade dysplasia including HPV infection represented the majority of the patients, ranging from 60.4 to 74%. Less than 16% of the patents in each age category showed no evidence of cervical dysplasia or HPV infection. Overall, patient ages were found to have no significant association with the severity of histological diagnosis (p=0.132), even after the patients were combined into two age groups (≤22 vs. >22) (P = 0.693).

Table 3 shows the relationship between Pap smear abnormalities and histological findings of 995 patients. Among the 596 (59.9%) patients with ASCUS, 167 (28.0%) had a histological diagnosis of HPV infection, 220 (36.9%) CIN1, 83 (13.9%) CIN2, and 42 (7.05%) CIN3. 372 patients (37.4%) had an LSIL smear with the following histological diagnoses: 91 cases (24.5%) with HPV infection, 174 (46.8%) CIN1, 45 (12.1%) CIN2, and 15 (4.03%) CIN3. Of the 27 (2.71%) cases with HSIL, 3 (11.1%) had HPV infection, 9 (33.3%) CIN1, 6 (22.2%) CIN2, and 8 (29.6%) CIN3. Overall, a significant relationship was found between the severity of cytological diagnosis and the severity of histological diagnosis (P<.0001).

Table 3 Relationship between cytological diagnosis and histological diagnosis

Cytological Diagnosis	Histological Diagnosis (biopsy or LEEP conization)					# Total cases (%)
	# ND (%)	# HPV (%)	# CIN1 (%)	# CIN2 (%)	# CIN3 (%)	
ASCUS	84 (14.1)	167 (28.0)	220 (36.9)	83 (13.9)	42 (7.05)	596 (59.9)
LSIL	47 (12.7)	91 (24.5)	174 (46.8)	45 (12.1)	15 (4.03)	372 (37.4)
HSIL	1 (3.70)	3 (11.1)	9 (33.3)	6 (22.2)	8 (29.6)	27 (2.71)

ND: negative for dysplasia or histological diagnosis; ASCUS: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesions; CIN1: cervical intraepithelial neoplasia 1; CIN2: cervical intraepithelial neoplasia 2; CIN3: cervical intraepithelial neoplasia 3

Table 4 Correlation of age, cytological diagnosis and histological diagnosis and HR-HPV load

Age (yrs)	# Cases	HR-HPV load		P value
		# H + M (%)	# L (%)	
≤22	256 (25.7)	173 (67.6)	83 (32.4)	0.046
23-30	312 (31.4)	180 (57.7)	132 (42.3)	
31-40	212 (21.3)	128 (60.4)	84 (39.6)	
>40	215 (21.6)	121 (56.3)	94 (43.7)	
≤ 22	256 (25.7)	173 (67.6)	83 (32.4)	0.007
>22	739 (74.3)	429 (58.1)	310 (42.0)	
Cytological diagnosis				
ND/ASCUS	596 (59.9)	302 (50.7)	294 (49.3)	<0.0001
LSIL	372 (37.4)	281 (70.4)	91 (29.6)	
HSIL	27 (2.71)	19 (75.5)	8 (24.5)	
Histological diagnosis				
ND	132 (13.3)	70 (53.0)	62 (47.0)	0.029
CIN1/HPV	664 (66.7)	398 (59.9)	266 (40.1)	
CIN2/CIN3	199 (20.0)	134 (67.3)	65 (32.67)	

HR-HPV load: viral load testing of high-risk types of human papillomavirus; H+M: cases of high viral load and moderate viral load; L: Low viral load; ND/ASCUS: No dysplasia and atypical squamous cells of undetermined significance on PAP; LSIL: low-grade squamous intraepithelial on PAP; HSIL: high-grade squamous intraepithelial on PAP; ND: No dysplasia; CIN1/HPV: low-grade cervical intraepithelial neoplasia 1 or HPV infection on biopsy; CIN2/CIN3: cervical intraepithelial neoplasia 2 or 3 on biopsy

Table 4 summarizes the correlation of HPV viral loads with patient age, cytology and histology findings. Overall, the age categorization was significantly associated with moderate to high viral load of HR-HPV (22 or younger: 67.6%, 23-30: 57.7%, 31-40: 60.4%, over 40: 56.3%; Chisq=8.00, df=3, p=0.046). When the 22 or younger group was singled out, such correlation was confirmed (Chisq=7.22, df=1, p=0.007). Moderate to high viral loads of HR-HPV were found to be significantly related to the histological grades of dysplasia (absent: 53.0%, low: 59.9%, high: 67.3%, Chisq=7.06, df=2, p=0.029). A strong correlation was identified between cytological diagnosis and high to moderate viral load (p<0.0001). While inspecting these findings in detail controlling for age, logistic regression analysis confirmed the association of HPV viral load with histological grade of dysplasia, even after adjusting for age. In particular, the high grade dysplasia (OR: 1.80, CI LL/UL: 1.14/2.83, p=0.011) but not the low grade dysplasia (OR: 1.33, CI LL/UL: 0.91/1.94, p=0.140) was significantly associated with HPV viral loads. Furthermore, 22 or younger was the only age group found to be significantly connected with moderate to high HPV viral load (OR: 1.60, CI LL/UL: 1.10/2.33, p=0.015) after adjusting for the degree of dysplasia.

Discussion

The female genital tract HPV infection is essentially the direct cause of uterine cervical cancers. However, the high prevalence of transient infections makes detection of the virus an inefficient marker for identifying patients at risk of high-grade dysplasia or cancer. Almost 70% of sexually active women are infected with HPV in their lifetime, but only a few will develop invasive cervical carcinoma [15, 16]. The cumulative incidence of HPV infection was 43% in 36 months in the college women. Younger age, ethnicity, multiple sex-partners and frequency of vaginal sex are major risk factors [15]. The medium duration of new infections is about 8 months and they are generally transient. All most all low-risk HPV infections and two thirds of HR-HPV infections are eradicated over two year period and women who have persistently positive HPV test results, particularly those in the high-risk group, are more likely to have LSIL and HSIL than those who revert to negative HPV tests [16]. In our study of 995 patients at Yale-New Haven Hospital, HR-HPV infection was strongly associated with young age, and a progressive decline of HR-HPV positivity was observed as age increases. Among 4 age groups, women of 22 years or younger was found significantly associated with moderate to high viral loads of

HR-HPV (p=0.046), similar to other reports [15, 16, 17]. Among the three factors including patient age, cytological diagnosis, and biopsy diagnosis, our study did not find any significant correlations between the patient age and the severity of cytological diagnosis (p=0.823) and/or the severity of histological diagnosis (p=0.132), even when patients are subdivided into two general groups (≤22 years and >22 years).

HPV molecular testing of cervical samples has become a standard practice to triage patients with borderline cytological findings and to guide clinical follow-up. However, the clinical and pathological applications of the quantitative information of HPV molecular testing, i.e. HPV viral load, are still controversial. The relationship between HR-HPV viral load and histological severity of cervical precancerous lesions has been debated considerably. Some studies have found that different levels of HR-HPV correlated with grades of CIN and invasive squamous carcinoma [2, 3, 4, 5, 6] and a high load of HPV-16 was found to be positively associated with the severity of cervical lesions [1, 10, 11, 12, 13]. A few studies reported that HR-HPV load, measured by Hybrid Capture II, correlated significantly with not only the severity but also the size of CIN [3, 7]. However, others have reported no significant correlations [7, 8, 9]. Using the Hybrid Capture II assay, our study found that moderate to high HPV viral loads were significantly related to high-grade dysplasia in biopsy (p=0.029). Logistic regression analysis further confirmed such association of HPV viral load with histological high-grade dysplasia, even after adjusting for age. Among patients who had high-grade dysplasia in their biopsies, the group of 22 years old or younger was the only age category found to have a significant correlation with a moderate to high HPV load (p=0.007).

The majority of HPV infections in younger women were transient and usually cleared in 7.5–14 months [15, 16, 17]. Moscicki reported that 70% young women with a positive HPV test will become negative within a 24-month period [17]. Persistent positive tests with HR-HPV types represented a significant risk for the development of HSIL in several prospective longitudinal studies [5, 18]. A high viral load resulting from productive viral replication might support viral persistence. It seems that tumor development is highly dependent on the initial immune response to the virus as well as to long-term high viral load. Viral clearance fluctuates depending on whether the patient has regressing or persistent HPV infection. Women with a high viral load are at increased risk for CIN2 and CIN3 and have a diminished chance of both viral clearance and cytological regression [1]. Women with low levels of HPV load were more likely to clear their infection than those with high viral load and infections with high levels of HR-HPV were reported to be more likely to persist, in particular if HPV-16 is the infectious agent [15]. Our findings and those of others are clearly in contrast to some notable reports where viral load was not found significantly correlated with the severity of cervical lesions. Case selection, specimen collection and HPV detection methods may explain some of the discrepancies. Digene HCII determination of HR-HPV has been well accepted for clinical practice and is FDA approved. Digene Cervical Sampler is a standardized specimen collecting procedure targeting the cervical transformation zone. Quantitation by HCII is highly reproducible with a four log analytical range, which was confirmed by our study as well (data not shown).

In conclusion, our data has confirmed that infection with high viral loads of HR-HPV is significantly associated with the high-grade CIN, particularly in the young age group (22 years old or younger). HPV viral load should be reported in the routine molecular HPV testing to provide additional useful information for evaluation of colposcopic biopsies and clinical follow-up of the patients. With the advent of HPV vaccines [22, 23, 24], our findings should advocate the current vaccination campaign to further reduce the global incidence of cervical cancer.

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