Original Articles

Relationship of human papilloma virus multiple genotype infection with patient's age and type of cervical lesion

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Summary

Purpose of investigation: To document the prevalence of infection by multiple genotypes of the human papilloma virus (HPV) in patients with cervical pathology in a study population, and to determine the relationship between multiple genotype infection, age of the patient, and the type of cervical pathology. *Materials and Methods:* Prospective, cross-sectional descriptive study. A total of 1,007 patients were recruited among women seen at the cervical pathology clinic of Sant Joan de Déu University Hospital in Barcelona (Spain) between January 2003 and March 2011. Statistical analyses were done with SPSS v.19 software. Differences between groups were considered statistically significant at $p < 0.05$. *Results:* There was 28.3% of the women (286 cases) that were infected by multiple HPV genotypes. The mean number of genotypes identified was 2.52 (range 2 to 8). Mean age of the patients with multiple genotype infection was 32.31 years, and mean age of the patients with single genotype infection was 37.27 years ($p < 0.001$). The prevalence of infection by multiple HPV genotypes was 28% in patients with cervical intraepithelial neoplasia grade 1 (CIN 1) and 33% in patients with grade CIN 2-3 lesions, and both prevalence rates were significantly higher than in patients with carcinoma (20%) (p=0.03). *Conclusions:* In the present study population the authors found no evidence of higher prevalence of multiple HPV genotype infection in women with carcinoma. Age of women with multiple infection was lower than those with single infection.

Key words: Human papillomavirus infection; Cervical cancer; Cervical intraepithelial neoplasia; Grade 1, 2, 3; Multiple infection.

Introduction

Human papillomavirus (HPV) is one of the main risk factors for invasive cervical cancer [1-3]. The genotypes involved most frequently are related with HPV 16 and HPV 18 [4-6] and are associated with approximately 70% of all cancers of the cervix, 50% of all high-grade intraepithelial neoplasias of the cervix, and 25% of all low-grade intraepithelial neoplasias of the cervix. However, other HPV genotypes are associated with high risk (HPV 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, 70, and 85) or probable high risk (HPV 53, 66), and also play an important role in cervical pathology and cervical cancer [7]. The prevalence of simultaneous infection by more than one genotype varies widely from 10% to 80% depending on the population studied [8-13].

It is currently unclear whether multiple HPV genotype infection is predictive of the severity of the cervical lesion. Controversy continues regarding the possible mechanisms through which multiple genotype infection might increase the risk of cervical intraepithelial neoplasia grade 2 or 3 (CIN 2-3) lesions or carcinoma [10, 11].

The aims of this study were to document the prevalence of infection by multiple HPV genotypes in patients with cervical pathology in a study population, and to shed light on how multiple genotype infection is related with the patient's age and with type of cervical pathology. Its interest remains in describing the authors' own population and own prevalence rates.

Materials and Methods

Study population

Information for this prospective cross-sectional descriptive study was gathered for a total of 1,007 patients, with a mean age of 35.8 years (range 14–73), seen at the cervical pathology clinic of Sant Joan de Déu University Hospital in Barcelona (Spain) between January 2003 and March 2011 [14]. All women were referred to the present hospital because of cytological alterations. The authors began collecting data from each patient at the moment of their first visit at the cervical pathology unit and it continued during the following visits and treatments.

The inclusion criteria were any alterations found on cervical cytology, and documented work-up at the present cervical pathology clinic.

Data collection

Cytological studies were done for all patients and the findings were classified according to the Bethesda Classification as atypical glandular cells of undetermined significance (AGC-US), atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial (LSIL), and high-grade squamous intraepithelial lesions (HSIL). All women were also examined by colposcopy with an aqueous 3%–5% acetic acid solution. The authors used the classification proposed in Barcelona by the International Federation for Cervical Pathology and Colposcopy in 2002 [15]: normal findings, abnormal findings (epithelial acetowhitening,

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punctuation or mosaicism, iodine-negative, atypical vessels), findings suggestive of invasive cancer, and unsatisfactory colposcopic examination. In all women the diagnosis was confirmed histologically. Samples were obtained by colposcopically-guided punch biopsy from all areas of the cervix with atypical colposcopic findings. The biopsy specimens were fixed in formalin, analyzed by a pathologist, and classified as follows: negative, CIN 1, 2 or 3, carcinoma, and adenocarcinoma.

HPV genotyping by PCR

Cervical scrapes were obtained with a cotton brush and transported at room temperature to the molecular microbiology department for HPV genotyping. Cytology was conventional and no medium was used to transport the cervical specimens. During the study period two techniques—line probe assay [LiPA] and microarray—were used consecutively. For LiPA assays, cervical swabs for DNA extraction were obtained with a commercial kit and eluted to a final volume of 200 µl. For microchip array assays, DNA was extracted with a proteinase K lysis solution (20 mg/ml). The purified DNA extracts were stored at −20 °C.

The LiPA assay was based on the reverse hybridization principle and provides type-specific genotype information for 25 different HPV genotypes (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74) simultaneously. Amplification of HPV DNA was based on the SPF10 PCR primer set, which amplifies a fragment of only 65 bp within the L1 open reading frame (ORF) region. Part of the human beta-globin gene (268 bp) was amplified in each sample as a control. Line probe assays with SPF10 were done with 10 µl of the DNA extract in a final reaction volume of 100 µl.

The microchip array assay detected infections and coinfections by up to 35 of the most relevant HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, and 89) in different sample types. The system was based on a low-density microarray attached to the bottom of a classical 2-ml Eppendorf tube. For DNA amplification a reaction mixture was used which amplifies a 450-bp fragment within the L1 ORF region. A 892-bp fragment of the human *CFTR* gene was amplified in each sample as a genomic DNA control. To avoid false negative results, an amplification control was added to the reaction mixture. The control used for the genotyping was the control of each of the kits.

In the present study HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, 70, and 85 genotypes were considered high-risk and HPV 53 and 66 probable high risk, on the basis of recently published studies. However, the present sample included no women with the HPV 26 genotype. The HPV 6, 42, 84, 61, 11, 54, 81, 43, 44, 62, 71, and 74 genotypes were considered low-risk in the present sample.

Statistical analysis

All data were analyzed with SPSS software (v. 19). The authors used Student's *t* test for quantitative variables when the data were distributed normally, and the Mann–Whitney U test when normal distribution could not be confirmed. Comparisons for qualitative variables were analyzed with the chi-squared test. Analysis of variance was used for comparisons involving more than two samples. The results were considered statistically significant if the *p* value was < 0.05.

Results

Of the patients included in this study, 48.7% (486) of them were diagnosed as having CIN 1, 47.3% (476) as CIN 2/3, and 3.7% (37) had carcinoma. The high percentage of patients with CIN 2/3 is due to all women that were referred to the present hospital due to cytological alterations from their primary health centers and most patients with transitory CIN 1 were not referred. Most women (77.9%, 686) had received surgical treatment, of whom 23% were treated with large loop excision of the transformation zone, and 54.9% underwent conization (cone biopsy); 9.3% underwent hysterectomy. A total of 740 women (73.2%) had HPV infection, among whom 86.4% (639) had a high-risk HPV genotype.

In the present sample, 28.3% of the women (286 cases) were infected by multiple HPV genotypes (included high-risk and low-risk HPV). Many patients with high-risk HPV genotype infection were infected with multiple HPV genotypes (43.7%), whereas among patients with low-risk HPV genotype infection, only 1.9% (nine cases) were infected with more than one genotype ($p < 0.001$). Among women with multiple HPV genotype infection, the mean number of genotypes per patient was 2.52 (range two to eight). Two-thirds of multiple infections (66.1%) involved two genotypes, 23.4% involved three, 5.25% involved four, 3.5% involved five, 1% involved six, 0.3% involved seven, and 0.3% involved eight genotypes.

Mean age of the patients with multiple genotype infection was 32.31 years, and mean age of the patients with single genotype infection was 37.27 years ($p < 0.001$). The age of the patients is considered at the time of diagnosis of cervical pathology. Mean age in women with HPV infection by

Table 1. — *Mean age of patients infected by different numbers of HPV genotypes.*

Number of genotypes	Mean age of patients (years)	*p*
2	32.90	0.44
3	32.03	
4	31.87	
5	26.80	
6	28.67	
7	23	
8	22	

Table 2. — *Frequency of multiple HPV genotype infection depending on the type of lesion.*

Type of lesion	Infection by one HPV genotype (number of patients and percentage)	Infection by multiple HPV genotypes (number of patients and percentage)	Total	*p*
CIN 1	275 (72%)	107 (28%)	382	0.03
CIN 2-3	309 (67%)	150 (33%)	459	
Squamous cell carcinoma	30 (80%)	7 (20%)	37	
Adenocarcinoma	2 (66.6%)	1 (33.3%)	3	
Total	619	265	881	

Table 3. — *Frequencies of patients infected by different numbers of HPV genotypes depending on the type of lesion, considering only patients with coinfection.*

Type of lesion	Number of genotypes							*p*
	2	3	4	5	6	7	8	
CIN 1	63.4%	24.8%	8.9%	3%	0%	0%	0%	0.98
CIN 2-3	64.7%	25.3%	3.3%	3.3%	2%	0.4%	0.4%	
Carcinoma	71.4%	14.2%	14.2%	0%	0%	0%	0%	
Total	172 (65.2%)	65 (24.6%)	14 (5.3%)	8 (3%)	3 (1.1%)	1 (0.4%)	1 (0.4%)	

two genotypes was 32.90 years, and mean age decreased as the number of infecting genotypes increased: mean age among women in whom eight genotypes were identified was 22 years. The differences between mean age according to the number of genotypes were not significant ($p = 0.44$), most likely due to the small size of the subgroups with coinfection by six, seven or eight genotypes (Table 1).

The prevalence of multiple HPV genotype infection was 28% in patients with a diagnosis of grade CIN 1 and 33% in those with a diagnosis of grade CIN 2-3. These prevalence rates were significantly higher than in patients with carcinoma (20%) ($p = 0.03$, Table 2).

Among patients with coinfection, the prevalence of coinfection by two HPV genotypes was 63.4% in patients with grade CIN 1, 64.7% in those with grade CIN 2-3, and 85.7% in those with carcinoma ($p = 0.98$, Table 3).

Discussion

In the population studied, the prevalence of infection by multiple HPV genotypes was 28%, a lower figure than in earlier studies [8-13]. Variability in the prevalence of multiple HPV genome infection may be explained by methodological differences between studies. Because different tests are used to detect HPV, comparisons across studies are problematic. In addition, differences in the characteristics of study populations may account for the variability, because infection by multiple genotypes is influenced by differences in geographic, demographic, and clinical factors [11, 12].

The prevalence of coinfection was higher among women infected with high-risk HPV genotypes (43.7%) than with low-risk HPV genotypes (1.9%). This finding is consistent with earlier results, as most series have reported a very low prevalence of multiple infection by low-risk genotypes, whereas most multiple infections involve high-risk HPV genotypes [8, 12-16].

The authors found a clearly higher prevalence of coinfection in younger women, and coinfection became less frequent as age increased. In addition, mean age in patients with coinfection decreased as the number of coinfecting genotypes increased. These results are fully consistent with those of earlier studies of the relationship between coinfection and age of the patients [8, 11, 13, 17-19]. All reports have noted a trend towards a higher prevalence of coinfection in younger patients. Young women are more likely to be infected with HPV per se, and to be infected by multiple genotypes, because multiple HPV genotype infections are closely related with sexual behavior [17]. Moreover, the inverse relationship between the prevalence of coinfection and the patients' age can also be attributed to acquired immunity which develops with the duration of exposure to HPV. This process may also explain why coinfection by a larger number of genotypes is more frequent at younger ages [20-25].

In connection with the relationship between coinfection and the type of lesion, a population-based study in Madrid (Spain) by Martin *et al.* [26] found that coinfection was more closely associated with grade CIN 1 lesions (45%) than with grade CIN 2-3 lesions (20%). These authors postulated that as the lesion progresses from low grade to high grade, genotypes that bear a high oncological risk persist while those with low oncological risk are eliminated. A study in an Italian population by Gargiulo *et al.* [16] also found a higher prevalence of coinfection among women with grade CIN 1 lesions (6.5%) than grade CIN 2-3 lesions (2.3%) or carcinoma (3.2%). Rousseau *et al.* [19] obtained similar findings: coinfection appeared in 23% of the women with grade CIN 1 lesions and 7% of the women with grade CIN 2-3 lesions. Muñoz *et al.* [27], in an international case-control study of cervical cancer, reported that multiple HPV genotype infections were not associated with a higher risk of carcinoma than single-genotype infections. Similarly, the SUCCEED study by Wetzensen *et al.* [18] at the University of Oklahoma found a higher percentage of single-genotype infection among cases of carcinoma (66%) than among lesions diagnosed as grade CIN 1 (24.7%). In the present population sample, patients with carcinoma had a lower prevalence of multiple infection than those with diagnosis of CIN 2-3.

It is currently unclear whether multiple HPV genotype infection is predictive of the severity of the cervical lesion. Moreover, the possible mechanisms by which multiple genotype infection may increase the risk of grade CIN 2-3 lesions or carcinoma are controversial [10, 11]. Some authors [8, 10, 11, 17] have suggested that compared to single-genotype infections, multiple HPV genotype infections are associated with an increased risk of grade CIN 2-3 lesions and carcinoma. These researchers have postulated that multiple-genotype infections may increase the risk of grade CIN 2-3

lesions or carcinoma because they are associated with a notable increase in the duration of HPV infection.

The present authors observed no relationship between the number of genotypes involved in coinfection and the type of lesion, and coinfection by two genotypes was the most frequent type of HPV infection regardless of the type of cervical lesion. These results are consistent with the findings of some earlier studies [11, 16, 18].

Additional studies are needed to evaluate the possible effects of multiple-genotype HPV infection on the risk of progression of cervical intraepithelial lesions, and to shed light on the mechanisms involved in their progression.

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