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Patterns of genotype distribution in multiple human papillomavirus infections

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ABSTRACT

The relationship between severe-grade cervical lesions and clusters of human papillomavirus (HPV) genotypes in a taxonomic classification was surveyed in 232 women with previous abnormal cytology. HPV co-infections were clustered according to phylogenetic criteria. Multiple infections were detected in 22.0% of the entire sample. Clade A10 (represented by HPV-6 and HPV-11) appeared more frequently in multiple infections than clade A9, which was represented by five of the most common high-risk types, including HPV-16. Although HPV-16 was the most frequent genotype, it was not more prevalent in multiple infections. Abortion and two or more sexual partners were risk-factors associated with HPV co-infections. Severe cervical dysplasia was associated with co-infections with oncogenic types from different clades, with the association being significant for the high-risk clades A7 and A9.

Keywords Cervical cancer, cross-sectional study, genotypes, human papillomavirus, multiple infections, risk-factors

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INTRODUCTION

Human papillomavirus (HPV) infects the skin and mucosal surfaces, with the large number of different HPV types being associated with different sites. Approximately 30 specific types infect the genital area by sexual transmission [1]. HPV types are characterised by DNA similarity and high levels of genome conservation. Genital HPV types are classified as high-risk or low-risk, according to their association with benign or malign disease [2], and are included in super-group A, with HPV-6 and HPV-11 (benign types) being referred to as group or clade A10, while HPV-16 and HPV-18, which are the commonest high-risk types for genital cancers, are included in clades A9 and A7, respectively [3].

Individuals can be infected by several HPV types, since each clade comprises unrelated viruses. Populations at high risk for cervical cancer, and populations with high rates of human immunodeficiency virus (HIV) infection, tend to show higher proportions of infections with multiple types, as compared with populations that do not belong to these risk groups [4]. Epidemiological studies suggest that the immune response to HPV is type-specific [5], but there is no agreement concerning the influence of infection with multiple HPV types on cervical carcinogenesis. According to Trottier *et al.* [6], infections with multiple HPV types seem to act synergically in cervical carcinogenesis. In contrast, Levi *et al.* [7] reported that although infection with multiple HPV types was a frequent finding in Brazilian women infected with HIV, concomitant infection with three or more HPV types did not confer an additional risk of cervical dysplasia in comparison with single/double infections, and was not related to more severe immune suppression. Co-infection with multiple HPV types, and its implications for the development of efficacious HPV vaccines, is a subject of great interest [8].

In view of these contrasting findings, the aims of the present study were to analyse the clinical

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significance of multiple HPV infections in the progression to cervical lesions, to investigate their association with typical risk-factors, and to determine the relationship between severe-grade cervical lesions and individual HPV genotypes.

MATERIALS AND METHODS

Samples

Cervical samples were collected from 238 women between 2000 and 2005. Six samples failed to amplify in PCRs (β-actin amplification-negative; see below). Methods of recruitment and data collection for the study have been described previously [9]. In brief, patients attended the Cervical Pathology Service of Hospital Universitario Antonio Pedro (Universidade Federal Fluminense, Niterói, RJ, Brazil). These women were either referred because of previous abnormal cytology results from public health services, or attended the Cervical Pathology Service in the hospital for cytological screening. Colpocitology was performed at the first or subsequent visit, and biopsies were performed for women with abnormal cervical cytology. Cervical lesions were classified as normal, HPV infection (koilocytosis), atypical squamous cells of undetermined significance, low-grade squamous intra-epithelial lesions, high-grade squamous intra-epithelial lesions (HSILs, situ carcinoma), and squamous invasive carcinoma. The Ethics Committee of the Medical College at the university approved the protocol for collection of samples and for obtaining informed consent. Final diagnoses were the result of cytological/histological examination. Patients who were diagnosed with HSIL and cervical cancer were designated as cases. Patients with a normal/inflammatory cervix or who were carrying benign cervical lesions were included as controls.

DNA extraction

Samples were incubated for 4 h at 50°C in digestion buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA, pH 8.0, Tween-20 0.5% v/v) containing proteinase K at a final concentration of 400 mg/L. The samples were then extracted with phenol–chloroform–isoamyl alcohol (25:24:1). DNA was precipitated with one-tenth the volume of 0.3 M sodium acetate and three volumes of ice-cold 100% ethanol, washed with ethanol 70% v/v, air-dried and resuspended in 50 μL of sterile water.

PCR procedure

Consensus primers MY09/11, which amplify a 450-bp target within the L1 region of HPV, were used to detect generic HPV DNA [10]. Amplification was performed in 50-μL reactions containing 1× PCR buffer (Invitrogen, São Paulo, Brazil), 200 mM dNTPs, 1.5 mM $MgCl_2$, 50 pmol of each primer, 0.25 U of *Taq* polymerase (Invitrogen) and 5 μL of sample. PCRs comprised 35 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min. The β-actin primers Ac1 and Ac2 (0.1 pmol each) were used to amplify a 330-bp region of human DNA as an internal sample control [11]. Negative controls were included in each run. Products were analysed on agarose 1.3% w/v gels by comparison with a 100-bp DNA ladder after staining with ethidium bromide.

HPV clustering

HPV typing was performed using primers targeting the E6 gene DNA sequences of HPV-6, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-45 and HPV-58 [12], yielding 230-, 89-, 134-, 119-, 97-, 132-, 186- and 100-bp fragments, respectively. Negative controls were included in each run. HPV co-infections were clustered according to phylogenetic criteria as follows: clade A7—HPV-18 and HPV-45; clade A9—HPV-16, HPV-31, HPV-33, HPV-35 and HPV-58; and clade A10—HPV-6 and HPV-11 [3].

Statistical analysis

The strength of the association among HPV genotypes in a patient group and the association with cervical lesions were assessed by calculating ORs and 95% CIs. The statistical significance of results was analysed using the chi-square test for heterogeneity with Yates' continuity correction. Fisher's exact test and the Mantel–Haentzel procedure for trend were used, when appropriate. The significance level of tests was set at p 0.05.

RESULTS

The patients were aged 14–82 years (mean 38.2 years). Most (80.5%) were from Rio de Janeiro state, had a low income status (64.4%), and had either attended only elementary school or were reported to be illiterate (53.9%). The most prevalent ethnic group was non-white (57.8%). The number of lifetime sexual partners ranged from one to 30 (mean 3.0), with sexual activity beginning at an age of 10–29 years (mean 17 years). Most (58.5%) patients stated that they had one current sexual partner, and 29.0% reported that they had ungergone an abortion. In addition to HPV infection, 13.2% of the patients were suffering from other sexually transmitted diseases.

HPV infection was detected in 77.2% (179/232) patients, with multiple HPV infections being detected in 22.0% (51/232). The most prevalent HPV types, including multiple infections, were HPV-16 (54.7%), HPV-6 (24.6%), HPV-18 (16.2%), HPV-11 (7.8%), HPV-35 (7.8%), HPV-58 (7.8%) and HPV-33 (1.7%). The precise HPV type could not be identified in 11.2% of HPV-positive samples by the specific primers used; these were referred to as undetermined. No cases of infection with HPV-31 were detected. The prevalence of high-risk and low-risk HPV infections was 58.6% and 10.3%, respectively. Most (85%) of the HPV-positive women were infected with high-risk types.