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CLINICAL ARTICLE

Human papillomavirus among women with atypical squamous cells of undetermined significance in southern Brazil

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ABSTRACT

Objective: To determine the prevalence of atypical squamous cells of undetermined significance (ASCUS) and human papillomavirus (HPV) genotypes in a population in southern Brazil. *Methods:* In a retrospective cross-sectional study, the prevalence of ASCUS was determined among women aged 20–60 years who were referred to a private medical center in Caxias do Sul by a gynecologist for assessment of a cervical condition between January 1, 2010, and September 30, 2011. Histologic and cytologic samples were tested for HPV, and polymerase chain reaction (PCR) was used to genotype any HPV DNA identified. *Results:* Among the 250 included women, 25 (10.0%) had ASCUS. HPV DNA was found in 15 (60.0%) women with ASCUS and 115 (51.1%) of the 225 without ASCUS. Viral typing showed that 7 (46.7%) HPV-positive women with ASCUS had multiple infections with up to five different genotypes. Both low- and high-risk HPV genotypes were found in ASCUS samples; the most prevalent genotypes were HPV6/HPV11 (affecting 10 [66.7%] women), HPV51 (6 [40.0%]), and HPV16 (6 [40.0%]). *Conclusion:* ASCUS is not an indication of HPV infection. HPV screening and genotyping would benefit women with ASCUS, because treatment can be planned according to risk of carcinogenesis.

1. Introduction

Cervical carcinoma is a fairly common malignancy worldwide. It has been estimated that there will be 15 590 new cases of cervical carcinoma in Brazil in 2014 [1]. Mortality is particularly high in the south of Brazil [1].

The most effective way to prevent cervical cancer is to diagnose and treat the precursor lesions, known as cervical intraepithelial neoplasia [2]. Cytologic study of a cervical smear sample is considered the most cost-effective method of detecting precursor lesions of cervical cancer. A set of cellular modifications can be identified and classified according to the presence and degree of atypical cells. The well-known Bethesda System is used to classify changes in cells by specific cytologic criteria: abnormal results include inflammatory low-grade squamous intraepithelial lesions (LSILs), high-grade squamous intraepithelial lesions (HSILs), and invasive carcinoma. Indeterminate changes can also be observed that do not meet the morphological criteria for classification as premalignant lesions and are known as atypical squamous cells of undetermined significance (ASCUS) [3].

It has been reported that ASCUS mature squamous cells would have a 10% risk of progression to squamous intraepithelial lesion [4]. The risk of progression for ASCUS metaplastic squamous cells increases to 24%, and that for ASCUS immature metaplastic cells rises to 41% [4]. Thus, the diagnosis of ASCUS requires clear treatment definitions. However, the clinical management of women with ASCUS is particularly problematic because this diagnosis can indicate several different conditions, including reactive cell changes and the development of pre-neoplastic or neoplastic stages [5]. There is controversy regarding the most appropriate procedures to use to prevent ASCUS evolving into more advanced cervical lesions. The recommended clinical approach is to repeat cervicovaginal cytology, colposcopy, and biopsy [5].

The association between human papillomavirus (HPV) and cervical dysplasia is well established [6]. HPV affects mitotically active cells of the epithelial tissue of the cervix, where it gains access to the basal and parabasal areas from the metaplastic epithelium. The virus can lead to premalignant transformation and abnormal growth of cells on the surface of the cervix, which can then progress to cervical cancer. The relationship between HPV and carcinogenesis depends fundamentally on the viral genotype (high- or low-risk HPV), viral load, and viral persistence and integration with the host cell [7]. The geographic distribution and prevalence of genotypes within a population are directly related to the effectiveness of programs for the primary prevention of cervical neoplasia [2,8]. Prophylactic vaccines have reduced the prevalence

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of HPV6 and HPV11 (low risk), and HPV16 and HPV18 (high risk) in some countries [9,10].

In this regard, viral typing of HPV DNA by polymerase chain reaction (PCR) is a useful molecular evaluation tool. It is a very specific and safe approach that allows a reliable diagnosis of both clinical and subclinical HPV infections, especially in cases of latent infections such as ASCUS. Viral typing is the most sensitive method to determine the presence of low- or high-risk HPV genotypes [11]. Considering the impact of HPV infection, the aim of the present study was to determine the prevalence of ASCUS, in addition to the prevalence of HPV genotypes in samples positive for ASCUS, among a Brazilian population using data from a clinical pathology laboratory.

2. Materials and methods

In a retrospective cross-sectional study, data were analyzed from women aged 20–60 years who were referred to the private Medical Center of Pathology, Caxias do Sul, Brazil, by a gynecologist for assessment of a cervical condition between January 1, 2010, and September 30, 2011. The study was approved by the University Ethics Committee. Informed consent from the patients was not deemed necessary, because all data were anonymous.

Diagnosis of ASCUS or cytopathology diagnosed as indeterminate ASCUS was made via cervical smear screening. Collection of the smear consisted of ectocervical and endocervical scraping, and harvesting with an Ayre spatula and endocervical brush. The samples were examined by an anatomopathologist who classified them according to the Brazilian Nomenclature for Reporting and Cytopathology Bethesda System 2011 [3].

All women underwent colposcopy. Before the colposcopy examination, biological samples were collected via a cytobrush to determine whether HPV was present and, if so, for typing of HPV DNA. The cytobrush was placed in 500 μL of Tris-EDTA buffer solution and stored at 2 °C–4 °C until DNA extraction. DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. After extraction, the DNA was analyzed by two different PCR methods: nested multiplex PCR, and multiplex PCR followed by restriction fragment length polymorphism (RFLP) analysis.

Nested multiplex PCR was conducted with a combination of primers that amplify 630 base pairs (bp) from the region containing genes E6 and E7. The primers used for amplification were GP-E6-3F (GGGWGKKACTGAAATCGGT), GP-E6-5B (CTGAGCTGTCARNTAATTG CTCA), and GP-E6-6B (TCCTCTGAGTYGYCTAATTGCTC). These primers amplify a region of 38 most common types of HPV, including HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66,or HPV68. The amplification conditions of PCR reactions and nested PCR have been described previously [11].

Samples that tested negative for HPV DNA by nested multiplex PCR were then subjected to multiplex PCR followed by RFLP. The quantity and quality of the DNA sample were first tested by amplifying a segment of 268 bp of the human β-globin gene using primers PCO4 (CAACTTCA TCCACGTTCACC) and GH20 (GAAGAGCCAAGGACAGGTAC). Samples positive for the human β-globin gene were then tested for HPV with the PGM09 and PGMY11 primers, which amplify a 450-bp segment of the preserved region of the L1 gene of HPV [12].

HPV-positive samples were submitted to RFLP analysis as described by Bernard et al. [12].The amplified product was digested by the *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, *Pst*I, *Rsa*I, and *Sau*AIII enzymes to produce electropherotyping data that were compared with the prototypes of Bernard et al. [12]. Each analysis included positive and negative control samples. The positive control comprised HPV genotypes HPV6/HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68, which had been previously typed (Diagnosis Molecular Laboratory, University of Caxias do Sul, Brazil). pGEM (Promega) was used as a molecular weight marker. The digested product was analyzed by vertical electrophoresis in 4% polyacrylamide gel and stained with silver nitrate.

A sample size calculation based on previous studies indicated that a sample of 250 women would be sufficient for analyses [6,13,14]. Data were tabulated via SPSS for Windows version 19.0 (IBM, Armonk, NY, USA). Data were compared by Pearson χ^2 or Fisher exact tests as appropriate. $P < 0.05$ was considered to be statistically significant.

3. Results

A total of 250 women were included. The mean age was 32.5 years. Overall, 47 (18.8%) women were aged 20–24 years, 108 (43.2%) were aged 25–34 years, 68 (27.2%) were aged 35–44 years, and 27 (10.8%) were aged 45–60 years. ASCUS was recorded for 25 (10.0%) women. The highest prevalence of ASCUS was recorded among women aged 35–44 years (Table 1), but the association between age and ASCUS was not significant ($P > 0.05$). Among the 225 women without ASCUS, 70 (31.1%) had LSILs, 8 (3.6%) had HSILs, 133 (59.1%) had inflammatory cytology, and 2 (0.9%) had carcinoma.

The DNA analysis showed that 130 (52.0%) women had HPV infection. Among the 25 women with ASCUS, 15 (60.0%) tested positive for HPV DNA (Table 2). However, the association between HPV and ASCUS was not significant ($P > 0.05$). Molecular analysis showed that 25 (19.2%) of the 130 women with positive HPV samples had single infections; 105 (80.8%) had multiple infections. Among the 15 HPV-positive women with ASCUS, 7 (46.7%) had multiple infections (Table 3). There was no significant link between ASCUS and whether HPV infection was single or multiple ($P > 0.05$).

Eighteen different HPV genotypes were identified in the samples by nested multiplex PCR (Table 4). Both low-risk genotypes (HPV6/HPV11, HPV42, HPV43, and HPV44) and high-risk genotypes (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV66, HPV68, and HPV82) were identified. The most prevalent HPV genotypes in women with and without ASCUS were HPV6/HPV11, HPV51, and HPV16 (Table 4).

High-risk HPV genotypes were recorded for 10 (66.7%) of the 15 HPV-positive women with ASCUS and 77 (67.0%) of the 115 without ASCUS (Table 5). Low-risk HPV genotypes were found in 13 (86.7%) HPV-positive women with ASCUS and 94 (81.8%) of those without ASCUS (Table 5). Therefore, women with and without ASCUS both showed high- and low-risk HPV genotypes, presenting no correlation between high-risk HPV and atypical cells (data not shown).

4. Discussion

The present findings showed that approximately 10% of women aged 20–60 years have ASCUS, although the frequency is increased in those aged 35–44 years (11.8%). Similar results were observed by Eltabbakh et al. [15], who reported an incidence of ASCUS of 10.2% in a population of 126 women in the USA. Another study [13] found a slightly higher incidence (15.4%) in a population of 714 women in Argentina.

After the initial diagnosis, women with ASCUS underwent further tests, which revealed that most cases were LSIL and HSIL (data not

Table 1
Prevalence of ASCUS, by age.[a]

Age group	With ASCUS	Without ASCUS
20–24 y (n = 47)	5 (10.6)	42 (89.4)
25–34 y (n = 108)	10 (9.3)	98 (90.7)
35–44 y (n = 68)	8 (11.8)	60 (88.2)
45–60 y (n = 27)	2 (7.4)	25 (92.6)

Abbreviation: ASCUS, atypical squamous cells of undetermined significance.
[a] Values are given as number (percentage).