

High prevalence and low E6 genetic variability of human papillomavirus 58 in women with cervical cancer and precursor lesions in Southeast Mexico

Jaqueline Canul Canche^{1/+}, Iván Rosado López², Nicolás G Suárez³, Gladis Colli Acosta⁴, Laura Conde-Ferrández¹, Thelma Canto de Cetina¹, María R González Losa¹

¹Laboratorio de Virología, Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán, Mérida, Yucatán, México ²Clínica de Displasias, Hospital General O'Horán, Secretaría de Salud, Mérida, Yucatán, México ³Clínica de Displasias, Hospital General, Secretaría de Salud, Chetumal, Quintana Roo, México ⁴Centro Anticanceroso, Mérida, Yucatán, México

Infection with some genotypes of human papillomavirus (HPV) is the most important risk factor associated with cervical cancer (CC). Throughout the world, HPV type 58 prevalence varies from one region to another; it is higher in women from certain countries in Asia and Latin America, such as China and Mexico. Although intratypic variants have been reported on a few occasions, our knowledge about HPV 58 genetic variation remains limited. Therefore, this work aims to (i) determine the prevalence of HPV type 58 amongst Mexican women with invasive CC or precursor lesions and (ii) identify HPV 58 sequence variants. One hundred and forty five colposcopy clinic patients were studied. Genotyping of HPV 16, 18 and 58 was determined by specific nested PCR and HPV 58 variants were detected by direct sequencing. The general prevalence of HPV was 51.7% (75/145). HPV 16 was found in 30.6% (23/75) and HPV 58 in 24% (18/75) of the patients. HPV 18 was not identified in patients with cervical intraepithelial neoplasia (CIN) grade I; it was only found in those with CIN II, with a prevalence of 6.8% (3/44). In patients with CC, the prevalence of HPV 16 and 58 was 78.9%. Regarding HPV 58 variants, 94.4% of the HPV 58 sequences were identical to the prototype strain, whereas one sample showed changes at a single nucleotide. This study demonstrates a high prevalence of HPV 58 and a low genetic variability of E6 sequences amongst Mexican colposcopy patients.

Key words: HPV 58 - variants - colposcopy clinic - PCR - CIN

Cervical cancer (CC) is the second most common cause of death due to neoplasia worldwide. Latin America is one of the regions with the highest incidence of this kind of cancer, representing around 13% of the world total (Arrossi et al. 2003). In particular, Mexico has a high incidence of CC, with a mortality rate of 10.2/100,000 inhabitants, representing a death every 2 h. Furthermore, in Southern Mexico, mortality rates due to CC are higher than in the Northern Region (SS 2005).

An important factor in the development of CC is infection with certain types of human papillomavirus (HPV) termed to be "high risk." Such infections are considered to be causal to the development of CC, given that the viral DNA has been found to be present in 97% of CC cases (Walboomers et al. 1999).

HPV is classified according to genome sequence similarity; a new type with a new number assignation is defined by a dissimilarity of 10% or more. Subtypes are defined by 90-98% sequence identity, while variants are defined when the homology in L1 gene sequences is around 98-99% (De Villers et al. 2004, Bernard 2005).

Worldwide, HPV types 16 and 18 are present in 70% of CC cases, followed in frequency by types 31 and 33 (Bosch et al. 1995). Given the importance of HPV 16, the study of its variants has been exhaustive. Initially, in 1993, Ho et al. compared the sequences of the HPV 16 long control region (LCR) and identified 48 variants from samples collected in five continents, from 25 different ethnic groups. These variants were grouped by geographic origin as European, Asian, Asian American, African-1 and African-2 (Chan et al. 1999, Calleja-Macias et al. 2005). The study of HPV 16 variants has facilitated the reconstruction of the evolution of HPV 16 through phylogenetic trees, showing the distribution and spread of the virus through different host populations (Lizano & García-Carranca 1997).

Several studies have demonstrated the differential biological behaviour of HPV variants. For example, Asian American variants are usually found in younger CC patients and in severe lesions (González-Losa et al. 2004), while other variants have been associated with specific histological features (Berumen 2003).

Worldwide, HPV 58 is the 6th most frequent type, although some differences in prevalence have been found according to the geographic region studied (Calleja-Macias et al. 2005). The highest prevalence of HPV 58 has been observed in Asiatic and Latin American countries (Huang et al. 1997, Chan et al. 1999, Herrero et al. 2000, Camara et al. 2003, González-Losa et al. 2003). In Mexico, several studies have reported a higher prevalence of HPV 58 amongst women with CC and precursor

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+ Corresponding author: jaqueline.canul@uady.mx

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lesions than that reported worldwide (Montoya-Fuentes et al. 2001, González-Losa et al. 2003, Piña et al. 2006, Aguirre et al. 2007).

Quintana Roo and Yucatan are located in Southeast Mexico; a region presenting higher mortality rates due to CC than the national average (13.4 and 18.6/100,000 inhabitants, respectively) (SS 2005). However, in this region only one HPV genotyping report has been published that demonstrated the relevance of HPV 58 amongst women with CC or precursor lesions. The objective of the current study was to determine the prevalence and variants of HPV type 58 amongst Mexican women with CC or precursor lesions.

PATIENTS, MATERIALS AND METHODS

Patients - This study, which took place between January 2006-February 2007, included women attending the Anticancer Center in Merida, Yucatan, the Colposcopy Clinic of the General Hospital in Chetumal, Quintana Roo and the General Hospital O'Horán in Mérida, Yucatan. These public hospitals are reference centres for women without social security. All patients were referred to the colposcopy clinic for an abnormal pap smear. Cervical cells samples were collected before the colposcopy procedure, using a cytological brush with the same technique used in Pap smears. The samples were placed in a transporter medium (isotonic saline solution with 500 U/mL penicillin, 500 µg/mL streptomycin, 4 mg/mL gentamicin, pH 7.2) and stored at -20°C until their evaluation. During the colposcopy, a biopsy for histopathological diagnosis was performed.

Epidemiological data and clinical histories were assessed using a questionnaire that included sociodemographic information, sexual behaviour, contraceptive history and tobacco use. All women signed an informed consent agreement to participate in the study.

HPV DNA detection and typing - The DNA extraction procedure was performed using the Dneasy Blood and Tissue Kit (QIAGEN), according to the manufacturer's instructions and carried out by experienced personnel.

β-globin amplification was performed as a DNA quality control, using primers PC04 y GH20 (260-bp amplicon) (Saiki et al. 1988). Universal L1gene primers MY09 and MY11 were used to determine HPV infection (450-bp amplicon), according to standard protocols (Qu et al. 1997), including a negative control without DNA every five reactions.

Nested PCR was used to detect the presence of HPV16, 18 and 58 DNA in HPV-positive samples, amplifying a fragment of the E6 and E7 genes using a previously reported methodology (Sotlar et al. 2004). Positive controls were clinical samples previously typified by reverse line blot hybridisation. All PCR reactions were carried out in a DNA Engyne thermal cycler (DYAD) and amplicons were visualised in 8% acrylamide gels using a 100-bp molecular weight marker (Invitrogen).

Variants identification - HPV 58 specific amplicons were gel-purified using the QIAquick Gel Extraction kit (QIAGEN) and sequenced using the dideoxyterminal method with 3700 DNA Analysis equipment (Applied

Biosystems) in the Laboratorio Nacional de Genómica CINVESTAV-Irapuato, Mexico. The sequences were analysed using the bioinformatics software, BLAST, SeqEdit and SeqMan II (DNASTAR, Lasergene) and compared with the prototype sequence of HPV 58 (access NC_001443) (Kirii et al. 1991).

RESULTS

In total, 155 patients were sampled; 93.54% (145/155) of the samples were included after amplification with β-globin primers and the rest were excluded. Histopathological diagnostics of the studied samples were as follows: cervical intraepithelial neoplasia (CIN) I: 62% (90/145), CIN II: 14.4% (21/145), CIN III: 9.6% (14/145) and CC: 13.7% (20/145).

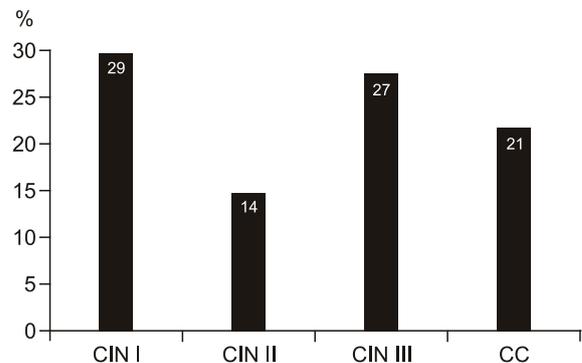
The general prevalence of HPV found was 51.7% (75/145). The prevalence of HPV related to lesion grade was as follows: CIN I: 34.4% (31/90), CIN II: 66.6% (14/21), CIN III: 78.5% (11/14) and CC: 95% (19/20).

The general prevalence of specific types of HPV was as follows: HPV 16: 30.6% (23/75) and HPV 58: 24% (18/75). The presence of HPV 18 was not tested in CIN I samples, so the prevalence amongst the studied samples was 6.8% (3/44).

The prevalence of specific types of HPV according to lesion grade was HPV 16, CIN I: 19.3% (6/31), CIN II: 28.5% (4/14), CIN III: 18.1% (2/11) and CC: 57.9% (11/19). HPV 58, CIN I: 29% (9/31), CIN II: 14% (2/14), CIN III: 27.2% (3/11) and CC: 21% (4/19). HPV 18, CIN II: 21.4% (3/14), CIN III: 0 and CC: 0.

The epidemiological data for the HPV 58-positive women showed that 55.5% (10/18) were from Quintana Roo and 44.5% (8/18) from Yucatan. The average age was 40 years old and the average number of pregnancies and births was five and four, respectively. The average number of sex partners was two, 88.8% (16/18) had become sexually active before age 18 and 55.5% (10/18) used oral contraceptives.

The 18 274-bp amplicons obtained from the HPV 58 E6 gene were compared to the prototype sequence published by Kirii et al. in 1991, with the following findings: 94.4% (17/18) were identical to the prototype and 5.6%



Human papillomavirus (HPV) 58 prevalence found in HPV-positive samples, according to lesion grade. CIN: cervical intraepithelial neoplasia.

(1/18) were classified as variant. The variant found had a single change in nucleotide 418, from thymine to cytosine. However, this nucleotide change is silent, given that the codon in the variant and the prototype both code for cysteine.

DISCUSSION

The main objective of this work was to determine the prevalence of HPV 58 infection and to identify the E6 sequence variation. Worldwide, HPV 58 is reported in 3.4% of women older than 50 years with CC (Muñoz et al. 2003). In contrast, a high prevalence of HPV 58 amongst women with CC and precursor lesions in the Southeast of Mexico is reported here. Previous studies in this region have reported HPV 58 with a frequency of 28.2%, higher than type 16 and one of the highest frequencies reported worldwide (González-Losa et al. 2003).

In Mexico, HPV 58 has been found in different regions. In Northern Mexico, Giuliano reported a prevalence of 13.6% in high grade squamous intraepithelial lesions (HGSIL) and found that HPV 58 was the second most frequently found genotype (Giuliano et al. 2001). In Eastern Mexico, Montoya-Fuentes (2001) reported HPV 58 as the second (16%) and third (15%) most prevalent genotype in HGSIL and CC, respectively. In central Mexico, a study of women with HGSIL reported a prevalence of 18.6% for HPV 58 (Piña et al. 2006).

Although lower than that reported in Mexico, prevalences of HPV 58 that are higher than the worldwide average have been found in Latin America. In Brazil, HPV 58 was found in 12.5% of women with CC and precursor lesions, being the second most frequent genotype (Camara et al. 2003). In Costa Rica, the prevalence of HPV 58 was 10% in HGSIL, again the second most common type (Herrero et al. 2000). In Colombia, the prevalence found in healthy women was 5.1% (Soto-De Leon et al. 2009).

Several studies have shown high HPV 58 prevalence in China (from 5.9-23.4%) and it is an important genotype amongst women with CC and CIN (Huang et al. 1997, Liaw et al. 1997, Chan 1999, Hong et al. 2008). Recently, a meta-analysis published by Bao et al. (2008) about HPV genotypes in Asia included 25,368 women from nine different countries. In that report, HPV 58 was the second most frequently found in women with all grades of CIN and the third most frequent in women with CC. HPV 58 was amongst the five more prevalent genotypes in all studied countries with the exception of India and Iran.

As shown, HPV 58 prevalence varies worldwide; however, the results reported here and the results from research conducted in China are, to our knowledge, the highest ever reported in CC and precursor lesions in the world. Previous studies from our group already revealed the importance of this genotype in the Mexican Southeast. (González-Losa et al. 2004)

Worldwide, HPV 16 and 18 have been reported as the most important genotypes in CC, as both are found in 70% of CC samples. However, geographic variations have been reported (Bosch et al. 1995). In this work, HPV 18 was detected in CIN II, but not in more severe lesions. Previously, our group reported HPV 18 in 13.5% of the samples

studied (Gonzalez-Losa et al. 2004), which is higher than that found in this work. These differences could be explained by the fact that in the present study, CIN I samples were not tested for HPV 18 and by the higher sensitivity of reverse line blot hybridisation with biotinylated primers, which was used in the previous work.

In 2007 the utilisation of vaccines against HPV was authorised in Mexico. Presently there are two vaccines on the market, both containing virus like particles (VLPs) from HPV 16 and 18 and one of them also including HPV 11 and 6 VLPs. According to what has been reported internationally, it is expected that 70% of vaccinated women will not develop CC (Paavonen et al. 2007). Interestingly, in our study, HPV 16 was present in 57.9% of all CC samples analysed, which suggests that the current available vaccines would not have the expected impact in the studied population. In order to add evidence to this observation, research including a larger sample number is currently in progress.

Knowledge of intratypic molecular variation of HPV 58 is poor and not conclusive. Furthermore, the genomic regions analysed are not uniform, which limits the possibility of comparing the results. The study of E6 variants is relevant because some HPV16 E6 specific variants have been associated with a higher CC risk (Lizano & Garcia-Carranca 1997, Matsumoto 2000, Villa et al. 2000, Gonzalez-Losa et al. 2004), therefore, it is very important to evaluate if a similar association is also present in other high risk genotypes.

However, limited information on HPV 58 variants has been published. In Japan, Xin et al. (2001) studied 40 HPV 58 strains, 95% of which were identical to the prototype. Chan et al. (2002) analysed the variation in E6 and E7 sequences and found more genetic variation in E7 than in E6.

In 2005, 21 HPV 58 - LCR variants were reported amongst 101 samples collected from different countries (Brazil, China, Mexico, Scotland, Africa and United States). From these, four variants were present in women from Northern Mexico; however, E6 variants were not sought in Mexican samples (Calleja-Macias et al. 2005).

Our results show low genomic variability in the HPV 58 E6 sequence within Mexican women. 94.2% (1/18) of the samples were identical to the nucleotide sequence of the Japanese prototype. This is in agreement with other studies, which have shown low variability in E6 from HPV 58, in contrast to other HPV types (31, 35, 33, 35 and 52), which exhibit higher variability in this gene (Xin et al. 2001, Calleja-Macias et al. 2005). More recently, Raiol et al. (2009) studied five samples from Central Brazil and reported that E6 sequences were highly conserved, with only one nucleotide change found.

The study of HPV 16 variants has demonstrated a specific geographical distribution (Yamada et al. 1997); however, in the case of HPV 58, the results presented here show the predominance of the Japanese prototype in a Southeast Mexican population.

This work is the first analysis of HPV 58 E6 variants in Mexico. Despite the limited number of samples, the present work establishes the basis for further studies on this subject, with larger sample sizes and the analysis of longer sequence fragments.

It is essential to continue the study in the region and in the country and to include L1 and E7 genes in order to gather more information about HPV 58 variants in Mexico and their impact in CC development.

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