**UNIVERSIDAD SAN FRANCISCO DE QUITO**

**Colegio de Posgrados**

# HPV 16 European variant and HPV 58 linage A2 associated with cancer in Ecuadorian women

**María Lorena Mejía Castañeda**

**Sonia Zapata, PhD.,** Directora de Tesis

Tesis de grado presentada como requisito

para la obtención del título de Magíster en Microbiología

Quito, diciembre de 2014

Universidad San Francisco de Quito

Colegio de Posgrados

## HOJA DE APROBACIÓN DE TESIS

# "HPV 16 European variant and HPV 58 linage A2 associated with cancer in Ecuadorian women"

MARÍA LORENA MEJÍA CASTAÑEDA

Sonia Zapata, PhD. _____

Directora de Tesis

Gabriel Trueba, PhD. _____

Director de la Maestría en Microbiología

y Miembro del Comité de Tesis

Pablo Endara, MD. PhDc _____

Miembro del Comité de Tesis

Stella de la Torre, PhD. _____

Decana del Colegio de Ciencias

Biológicas y Ambientales

Víctor Viteri Breedy, PhD. _____

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Nombre: María Lorena Mejía Castañeda

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# DEDICATORIA

A las personas afectadas por esta enfermedad que decidieron ser parte del estudio.

A mi familia, cuyo apoyo es incondicional y eterno.

## RESUMEN

El Papilomavirus Humano (PVH) es la infección de transmisión sexual más común en el mundo, el mismo que puede producir neoplasias intraepiteliales y cáncer cervical y en el área anogenital. La detección de la distribución de los tipos de PVH entre lesiones cancerosas es necesaria para analizar el potencial impacto de las vacunas disponibles y la implementación de estrategias profilácticas y de cribado. La identificación molecular de variantes de PVH es una herramienta poderosa para revelar información epidemiológica y filogenética sobre los genomas circulantes en un área específica. Sin embargo, en Ecuador, existe información limitada sobre los tipos de PVH en lesiones cancerosas y aún más limitada sobre sus variantes. El objetivo de este estudio fue caracterizar los tipos de PVH responsables de las neoplasias asociadas al tracto genital en mujeres ecuatorianas que asistieron a un hospital en Quito. El PVH fue tipificado mediante el *21 HPV GenoArray Diagnostic Kit*. Un total de 167 muestras pre-cancerosas y cancerosas fueron analizadas y un 86% fueron PVH positivas. Los tipos más comunes fueron PVH16 (42%) y PVH58 (31%) en todos los grados de lesión. PVH18 estuvo presente únicamente en 4 muestras (2.4%). Las muestras positivas para PVH16 y PVH58 fueron analizadas al amplificar y secuenciar los genes L1 y E6. Los resultados de estos análisis indicaron que 14 (93%) muestras PVH16 positivas pertenecieron al linaje Europeo y únicamente 1 (7%) al linaje Asia-Americano; 10 (83%) de las muestras positivas para PVH58 se agruparon con el sublinaje A2, 1 (8%) perteneció al sublinaje A3 y 1 (8%) al linaje C. Los resultados de este estudio podrían advertir un potencial impacto menor en la reducción de cáncer cervical del uso de las vacunas contra PVH disponibles en el Ecuador.

## ABSTRACT

Human Papillomavirus (HPV) is the most common sexually transmitted infection worldwide which can cause intraepithelial neoplasias and cancer in cervix and anogenital areas. The detection of HPV types distribution among cancer lesions is necessary in order to analyze the potential impact of the available vaccines and the implementation of prophylactic and screening strategies. The identification of HPV molecular variants is a powerful tool for revealing epidemiologic and phylogenetic information about circulating HPV genomes in a specific area. However, in Ecuador there is limited information about HPV types in cancerous lesions and there is even more limited data about genome variants. The goal of this study was to characterize the HPV types responsible for malignancies associated with the genital tract in Ecuadorian women attending a major hospital in Quito. HPV was genotyped by the 21 HPV GenoArray Diagnostic Kit. A total of 167 cancerous and precancerous samples were analyzed and an 86% were HPV positive. The most common types were HPV16 (42%) and HPV58 (31%) in all grade lesions. HPV18 was found only in four samples (2.40%). Samples positive to HPV16 and HPV58 were further analyzed by amplifying and sequencing E6 and L1 genes. These analyses indicated that 14 (93%) of the HPV16 detected belonged to the European lineage and only 1 (7%) to Asian-American lineage; 10 (83%) of HPV58 grouped in A2 sub-lineage, 1 (8%) belonged to A3 and 1 (8%) to lineage C. Results of this study may inform about the potential lower impact on reducing cervical cancer of the bivalent and tetravalent HPV vaccines currently in use in Ecuador.

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# INTRODUCTION

## *Background*

Papillomaviruses are double-stranded DNA viruses that belong to *Papillomaviridae* family [1], classified in 29 genera [2]. Papillomavirus (PV) genomes have been found in humans (120 types), reptiles (2 types), birds (3 types), marsupials and other mammals (64 types) [2] suggesting that PV evolutionary history goes back to 300 million years ago [3]. Evolution on this family has been mainly based on mutations, deletions and insertions and not on recombination, consequently variations have not a genetic distance correlation [3 4]. The mutation rate on PV genomes is related to the host genome rate they infect since the virus uses its host machinery for replication [4]. Lineage fixation is the term used to describe PV speciation and describes how multiple SNPs (single nucleotide polymorphisms) and indels (insertions and deletions) become fixed within papillomavirus lineages. The amount of these variations over time leads to speciation [3]. When nucleotide divergence between genomes is 10% or more, 2-10%, and less than 2%, they are considered to be different types, subtypes, and variants, respectively [3 5]. There are more than 160 types capable of infecting humans [6] that belong to different genus. However, alpha-papillomavirus comprise PV types that infect mucosal and cutaneous lesions in humans and primates with unique pathogenic activity [5].

## *HPV and cancer*

More than 40 types are grouped in the alpha genus which can infect mucosal tissue in the anogenital area [3 7] and are involved in benign and malignant outcomes. These types can be grouped according to its levels of association with the progression of cancer. “Low risk” (LR) HPV types are 6, 11, 42, 43, 44 and 81 among others [8] and are generally associated to condyloma acuminate, respiratory papillomatosis and low-grade cervical intraepithelial lesions. They are rarely found on high-grade lesions [7]. However, “high-risk” (HR) types are causative of squamous and glandular high-grade intraepithelial lesions and cancer [9 6] including cervical (100% of association between HPV and lesion), anal (90%), vulvar (40%), vaginal (40%), penile (40%) and oropharyngeal cancer (12%) [7]. HR HPV types are 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66 and 68 among others [8].

Cervical cancer is the fourth cause of female cancer deaths worldwide [10], while in Latin American it is the second [11] and in Ecuador it reaches the first most important type of cancer mortality in women of all ages [12]. It is noteworthy that HPV is essential but not sufficient to cause cancer. The infection may be affected by host and viral genome variations, the host immune system and environmental factors [3]. HPV16 and HPV18 are the two most prevalent types among women with cervical lesions around the world accounting for 70% of cases [7]. However, in high-grade lesions HPV16-related types (such as HPV31, 52, 58, 33) are more important, especially in less developed countries where HPV58 is the second most frequent type while HPV18 ranks in fourth place [10] (Fig. 1).

***HPV Biology***

Human Papillomavirus (HPV) is a circular, non-enveloped, double stranded DNA virus that codifies 6 early expression genes (E1, E2, E4, E5, E6 and E7), protected by an icosahedral capsid composed of L1 (major capsid protein) and L2 (minor capsid protein) [9] (Fig 2). Early genes codify non-structural proteins responsible for viral functions. E1 works as a DNA depending ATPase and ATP depending helicase to regulate viral replication and transcription. E2 mediates episomal DNA dispersal during cell division. Protein E4 associates and destabilizes cytokeratins networks allowing koilocytes formation [13]. It is also believed that regulates viral cycle by modulating host cell division cycle [14]. Protein E5 joins to membrane proteins, like growth factor receptors, and mediates cell transformation [13]. E5 can also prevent apoptosis resulting from DNA damage [1]. However, it is noteworthy that a part of E5 is deleted when the episomal viral genome becomes integrated [1]. E6 and E7, known as oncogenes, are the most important genes that affect cell proliferation [13] by blocking the activity of tumor suppressors [1]. E6 joins to and marks P53 protein and promotes its degradation, thus resulting in apoptosis resistance and chromosomal instability. E6 is also capable of activating a catalytic subunit of telomerase, which is important for cellular immortalization. E7, however, inactivates Rb protein and also induce centriole amplification causing aneuploidy that contributes to carcinogenicity [1].

***Infection***

HPV requires epidermal or mucosal proliferative epithelial cells in order to establish an infection [1 6]. Viral gene expression is suppressed in these cells causing a greater and lateral proliferation of infected cells [1]. About 91% of infections are naturally cleared by

the immune system within two years [7] [9] but in rare cases the infection persists during decades causing different grades of intraepithelial lesions and cancer [7], even though most low-grade lesions may regress spontaneously [6] by humoral and cellular immune responses against viral infections [1]. Certain modifications of cellular genes affecting antigen presentation or the inability to successfully suppress viral oncogene transcription or functioning may cause persistent HPV infections, which is the most important risk factor for developing cancer [1].

Besides high-risk HPV and viral load, there are other factors that contribute to malignancy [1]. Viral genome integration seems to play an important role in HPV pathogenesis. In high grade lesions, viral genome becomes integrated, deleting part of gene E2, which regulates viral gene transcription, and causing a greater E6 and E7 expression and viral proliferation; while in low grade lesions, viral genome stays in episomal form and E2 protein remains intact [15]. However, not all cancers have HPV genome integrated and it has been found integration of viral genome in HPV infections with normal cytology [16].

***Co-infections***

Multiple HPV types infections are very common, especially in women with no cytological abnormalities [17] [18]. Presence of one HPV type is associated to a higher risk of acquiring other HPV types [17], since it is the most common sexually transmitted virus. However, Thomas *et al* in their study suggested that there is no more likelihood of acquiring two specific HPV types than any other two types [19]. It was previously thought that a co-infection may represent an increased risk for cancer but now there is evidence that the risk for persistence or cancer is independent of multiple types presence [17] [18].

***Epidemiology***

Some authors have proposed that complex interactions between HPV types and HLA haplotypes may explain the differences in circulating HPVs in different regions of the world [20]. For example, some countries show a low frequency of HPV18 and it may be because this HPV type seems to be uncommon in South America (only 5% of HPV infections) [20] and HPV18 has been detected in 5.68% of HPV positive samples in Ecuadorian women (unpublished data). Whereas in some regions in Asia[21] [22], México[23], Nicaragua [24] and Brazil [25] show a high frequency of HPV58 in lesions of all grades.

With specific viral geographic dispersion and human closely evolution over time, HPV types have evolved into variant lineages and sub-lineages (1.5-10% and 0.5-1.4% of nucleotide divergence, respectively), with different association to higher risk of persistent infections and progression to cancer [4 26]. HPV16 variants may vary in risk but they are all carcinogenic, while some HPV types with moderate oncogenic activity (such as 31, 52, 56 or 58) may be a mixture of strongly carcinogenic lineages and variants with very weak carcinogenicity [26]. Furthermore, for some types, like HPV16 and HPV18, variants are associated to specific geographical locations and even to ethnic populations but for other types this association is not well established [3].

HPV16 variants are probably the best studied and characterized. One the first studies to be published in 1993 suggests that principal human races, such as Africans, Caucasians and East Asians, coevolved with papillomaviruses and it seems that various events of viral speciation took place in Africa. This research was the first study to establish a specific geographic distribution for HPV 16 and HPV18 variants [27]. One point to consider is that the authors calculated the HPV18 mutation rate and they suggested that it would be an approximation of that for HPV16 [27]. However, in latter studies, it was confirmed that mutation rate in HPV does not have a correlation with genetic distance [1 3]. There are 4 lineages (A, B, C and D) and 9 sub-lineages (A1-A4/B1-B2/D1-D3) [3] with a specific geographic distribution. Lineage A is known as the European branch, sublineage A2 is termed specifically as German variants and A4 as East Asians. African variants belong to lineage B and C. Sub-lineage D1 are termed as North American variants while D2 and D3 as Asian-Americans [3]. The association of variants and its geographic location is relevant because Non-European HPV16 sub-lineages (especially Asian-American variants) tend to be more persistent [28], more aggressive and more carcinogenic than European counterparts [3 4]. Greater carcinogenicity may be associated to more efficient viral replication (higher copy number per cell), and greater p53 degradation by E6 protein of this specific variants [29] (Fig 3).

Little is known about HPV58 molecular variants around the world except for one study done in Asia with collaboration from 15 countries where the distribution worldwide of 401 isolates was assessed. They also established the identification of HPV58 lineages and sub-lineages [30]. HPV58 variants are classified in 4 lineages (A, B, C and D) and 7 sub-lineages (A1-A3/B1-B2/D1-D2) [4], but the evolutionary and pathological traits of all

variants require further study. Despite the lack of epidemiological information outside Asia, it is known that sub-lineage A2 seems to be the oldest lineage and probably disseminated with the early human migration into different parts of the world before other lineages emerged. This is because A2 was found in Africa, Asia, America and Europe while lineage D was not found in Asia and sub-lineage A1 was not detected in America [30]. About sub-lineage A3, two substitutions have been found to be associated to a higher risk of cancer: E7 T20I, which is located near RB protein joining domain, causes greater association to this protein, and E7 G35S, where a glycine is changed to serine, creates an additional phosphorylation site which may increase oncogenic activity [21] (Fig 4).

HPV distribution related specifically to cancer in Ecuador is not well defined [12]. However, there are some publications about HPV in anogenital samples [31] and cervical dysplasia [32] in Ecuadorian women. A pilot study published in 2008, worthy to be mentioned, genotyped HPV in 71 chronic cervicitis and pre-cancerous samples and its results suggested that HPV16 (64.5%) and HPV81 (29%) were the most frequently found, followed by HPV31, 53, 56 and 58. Besides, the authors also showed that 85% of HPV16 variants were European and 15% belonged to African-1 sub-lineage. No Asian-Americans or North Americans were found [33]. Despite the information given by the authors, Ecuadorian studies about HPV types on cancerous lesions are not available.

***Prophylactic measures***

In an effort to prevent 70% of cervical cancers worldwide, two vaccines target the most common HPV types (HPV16 and 18). Gardasil, a quadrivalent recombinant vaccine, developed by Merck, with HPV6, 11, 16 and 18 VLPs (virus like particles) and Cervarix, a bivalent recombinant vaccine, produced by GlaxoSmithKline, with HPV16 and 18 VLPs [7]. Both prophylactic vaccines work in a three-dose regimen, however, one dose could be as protective and effective as three doses [34].

According to WHO, at the end of 2012, 45 countries had implemented HPV vaccination programs [11], while in Ecuador it was introduced early this year on the Public Vaccination Program [35] for 9 to 11 years old girls in a two-dose regimen. Nevertheless, there is a nona-valent vaccine, produced by Merck, which is waiting to be approved in United States. The introduction of a nona-valent vaccine may reduce the combined incidence of high

grade lesions in cervical/vulvar/vaginal disease (caused by genotypes 6, 11, 16, 18, 31, 33, 45, 52 and 58) in 96.7% [36].

The detection of HPV types distribution among cancer lesions is important in order to analyze the potential impact of the available vaccines and to include more types in future prophylactic measures and screening methodologies. In this study, the distribution of 21 HPV types among pre-cancerous, including CIN II (Cervical Intraepithelial Neoplasia) and CIN III, and cancerous (in situ and invasive) lesions in Ecuadorian women were evaluated. The presence of lineages and sub-lineages from the two most frequent HPV types in mono-infection samples was also analyzed.

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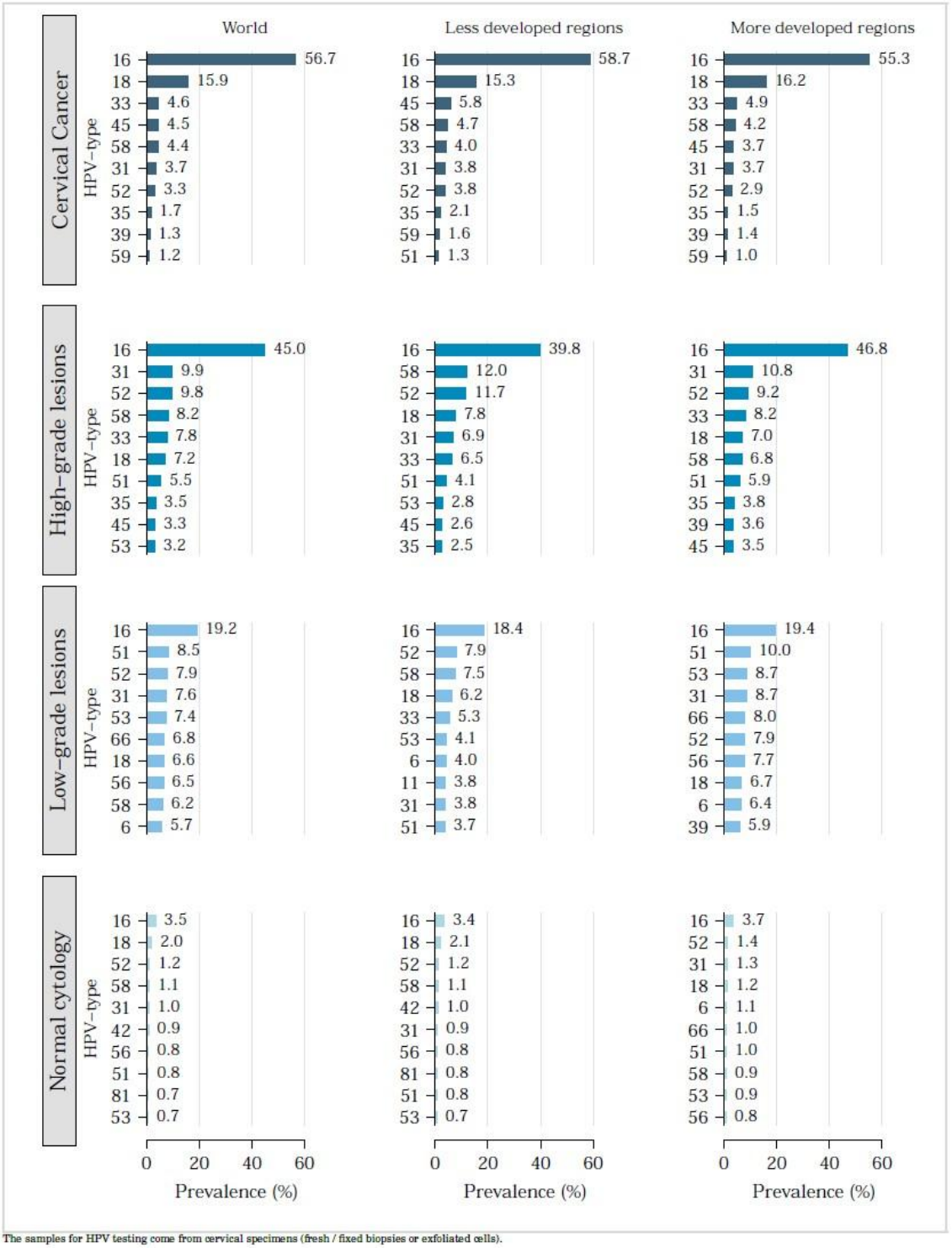
*Figure 1. Most frequent HPV types among women with normal cytology and cervical lesions worldwide compared to developing and developed countries (Bruni et al. 2014)*




The samples for HPV testing come from cervical specimens (fresh / fixed biopsies or exfoliated cells).

*Figure 2. Circular and integrated HPV DNA organization (zur Hausen 2002).*

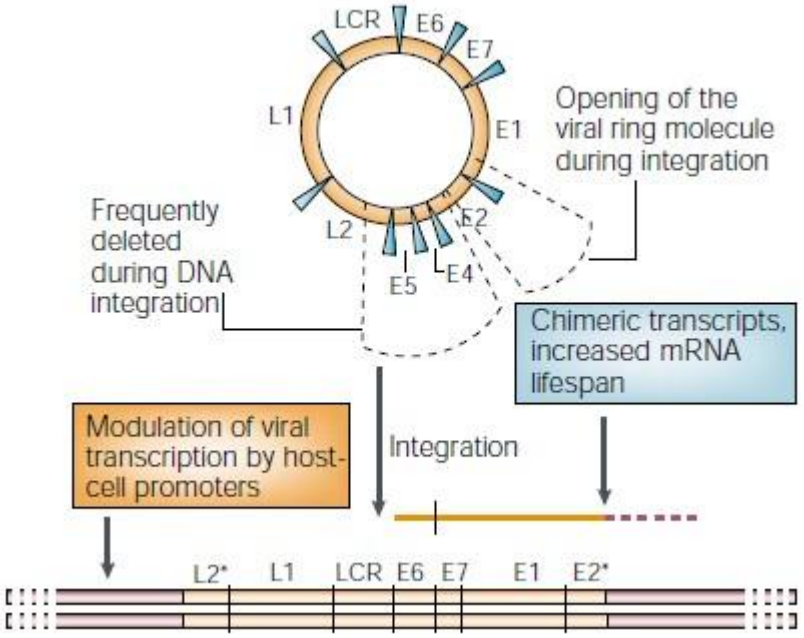



*Figure 3. HPV16 lineages and sublineages classification (Burk, Harari, and Chen 2013)*

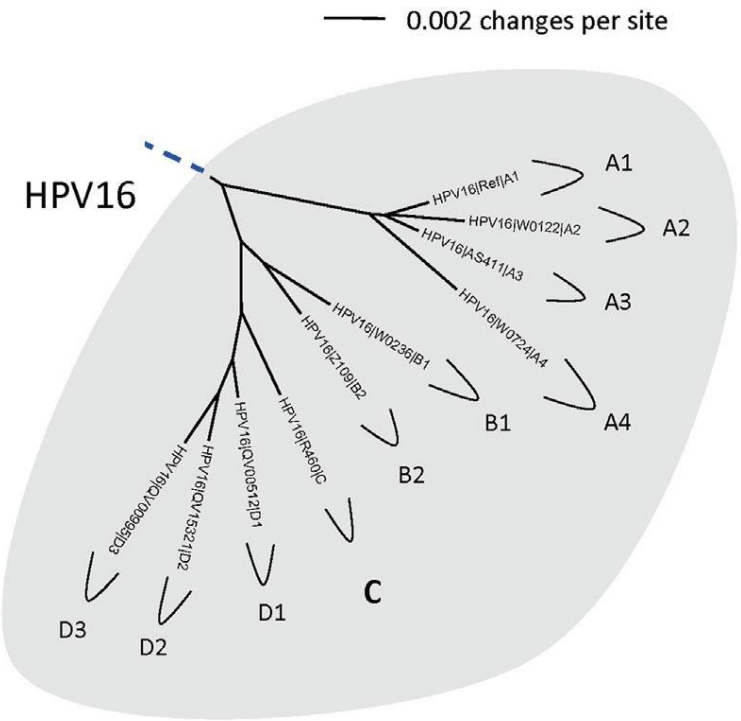
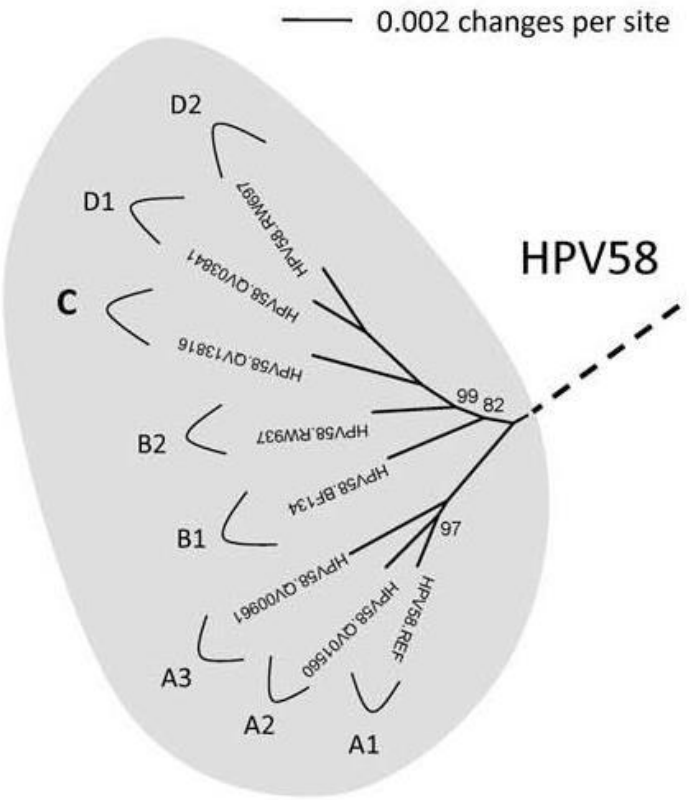



*Figure 4. HPV58 lineages and sublineages classification (Chen et al. 2011)*


0.002 changes per site
HPV58
D2
D1
C
B2
B1
A3
A2
A1
HPV58.RW637
HPV58.QV03841
HPV58.QV13816
HPV58.RW937
HPV58.BF134
HPV58.QV00961
HPV58.QV01560
HPV58.REF
99
82
97

# HPV 16 European variant and HPV 58 lineage A2 associated with cancer in Ecuadorian women

Lorena Mejía,[1] Diana Muñoz,[1] Gabriel Trueba,[1] Leopoldo Tinoco,[2] and Sonia Zapata[1] *

[1] Instituto de Microbiología, Universidad San Francisco de Quito, Diego de Robles y Pampite, Quito, Ecuador

[2] Unidad de Ginecología y Colposcopía, Hospital SOLCA, Av. Eloy Alfaro 5394, Quito, Ecuador

*** Corresponding author**

Sonia Zapata

Instituto de Microbiología, Universidad San Francisco de Quito

Diego de Robles s/n y Pampite

170157 Quito - Ecuador

E-mail: szapata@usfq.edu.ec

(593) 2971700 ext. 1427

**Shortened title:**

**HPV16 and HPV58 variants on cancer on Ecuador**

## ABSTRACT

Human Papillomavirus (HPV) is the most common sexually transmitted infection worldwide which can cause intraepithelial neoplasias and cancer in cervix and anogenital areas. The detection of HPV types among cancer lesions is necessary to analyze the potential impact of the available vaccines and the implementation of prophylactic and screening strategies. The identification of HPV molecular variants is a powerful tool for revealing epidemiologic and phylogenetic information about circulating HPV genomes in specific areas. However, in Ecuador there is limited information about HPV types in cancerous lesions and there is even more limited data about variants. This study's goal was to characterize HPV types responsible for malignancies associated with the genital tract in Ecuadorian women attending a major hospital in Quito. HPV was genotyped by the 21 HPV GenoArray Diagnostic Kit. A total of 167 cancerous and precancerous samples were analyzed and an 86% were HPV positive. The most common types were HPV16 (42%) and HPV58 (31%) in all grade lesions. HPV18 was found in four samples (2.40%). Samples positive to HPV16 and HPV58 were further analyzed by sequencing E6 and L1 genes. These analyses indicated that 14 (93%) of the HPV16 detected belonged to European lineage and only 1 (7%) to Asian-American lineage; 10 (83%) of HPV58 grouped in A2 sub-lineage, 1 (8%) belonged to A3 and 1 (8%) to lineage C. Results of this study may inform about the potential lower impact on reducing cervical cancer of the bivalent and tetravalent HPV vaccines currently in use in Ecuador.

**KEYWORDS**

HPV16 variants, HPV58 variants, Cancer, Ecuador

## INTRODUCTION

Human papillomavirus is responsible for 98% of cervical cancer cases [Khan et al. 2005] which is the fourth most important cause of cancer associated deaths in women worldwide [Bruni et al. 2014b]; in Ecuador it is the first cause of cancer deaths in women of all ages [Bruni et al. 2014a]. Human Papillomavirus (HPV) is a circular, non-enveloped, double stranded DNA virus, protected by an icosahedral protein capsid [WHO 2010], which belongs to *Papillomaviridae* family. Approximately 40 types (classified in the *Alphapapillomavirus* genus) can infect mucosal tissue in the anogenital area [Dunne and Markowitz 2006] and show different levels of association to cancer. Viral types known as "low risk" (LR) cause condyloma acuminate, respiratory papillomatosis and low-grade cervical intraepithelial lesions; those classified as "high-risk" (HR) can cause squamous and glandular high-grade intraepithelial lesions and cancer (cervical, anal, vulvar, vaginal, penile and oropharyngeal) [WHO 2010; Dunne and Markowitz 2006; Jong 2004]. However, 90% of high-risk HPV infections are cleared within two years by host immune responses [WHO 2010; Dunne and Markowitz 2006]. Types HPV16 and HPV18 are the two most commonly associated to cancerous lesions in cervix around the world (70% of cases) [Dunne and Markowitz 2006] and these types are covered by the vaccines used in many countries including Ecuador [WHO 2013; MSP 2014b].

Identification of genome variants could be very informative about epidemiology and phylogenetics on HPV and about the spread of the virus in specific areas. High risk HPV types have diverged into lineages and sub-lineages (1.5-10% and 0.5-1.4% of nucleotide divergence, respectively) displaying different levels of carcinogenicity [Z. Chen et al. 2011; Schiffman et al. 2010]. Besides, variants of HPV16 and HPV18, are associated to specific geographical locations and even to ethnic populations.

In this study, 167 pre-cancerous and cancerous lesions from Ecuadorian women were analyzed in order to determine types, lineages and sublineages of HPV associated with this pathology.

## MATERIALS AND METHODS

*Clinical samples*

Pre-cancerous and cancer biopsies were obtained from all patients attending to colposcopy consulting in SOLCA Hospital in Quito (reference oncologic hospital that receives patients from different regions of Ecuador) from October to December 2011 and from May 2012 to June 2013. Informed consent was obtained from all the participants, and the study was approved by the bio-ethics Committee of the Universidad San Francisco de Quito (USFQ) and the Teaching Department of SOLCA Hospital. Pathological tissue was collected by biopsy and split in two parts; one part was sent to SOLCA´s histopathology laboratory and another was preserved in 70% ethanol at -20°C for further processing. All of samples with high grade intraepithelial neoplasia (CIN II and CIN III) were included and subjected to DNA extraction.

*DNA extraction*

Total DNA was isolated by CTAB method described elsewhere [Saghai-Maroof et al. 1984] with some modifications. Biopsies on 75% ethanol were washed twice in 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM $KH_2PO_4$, pH 7). Each sample was split in half, one part was kept in a freezer at -20ºC and the other was cut into small pieces with a sterile scalpel and digested with 20 µl proteinase K (20 mg/ml) in 700 µl CTAB solution (2% CTAB weight/vol, 1.4 mM NaCl, 20 mM EDTA pH 8, 100 mM HCl pH 8) for 2 hours at 65°C with occasional mixing by inverting the 1.5 ml tubes. Chloroform/isoamyl alcohol (24:1 vol/vol) (700 µl) was added and the solution was vigorously mixed to form an emulsion that was centrifuged at 13, 300 x g for 5 min at room temperature. The aqueous phase (500 µl) was removed to a new tube with 1 ml of ethanol 100%, mixed by three-to-five inversions and kept at -20°C overnight. After a centrifugation step at 16, 110 x g for 10 min, the supernatant was discarded followed by

a washing step with 1 ml of 70% ethanol and centrifugation at 16, 110 x g for 10 min again. The supernatant was discarded and the tubes were dried at room temperature for 20 to 30 min. Finally, DNA pellet was re-suspended in 50 µl of TE buffer (10 mM Tris-HCl pH 8, 0.1 mM EDTA) and kept at -20°C for further processing.

*HPV genotyping*

The samples (1µl of total DNA) were genotyped by the 21 HPV GenoArray Diagnostic Kit (Hybribio Limited, Hong Kong, China) according to the manufacturer's instructions. The Hybribio's technique is based on E6 amplification and subsequent flow-through hybridization with specific probes within a membrane to identify 21 alpha HPV genotypes: 6, 11, 42, 43, 44 and 81 (low risk) and 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 (high risk).

*Molecular analysis*

Genes E6 and L1 from the HPV16 and HPV58 mono-infected samples were amplified using a PCR protocol described elsewhere [Sotlar et al. 2004] with some modifications. In brief, E6 PCRs were carried out in a final volume of 30µl containing 1X colorless reaction buffer, 1.6mM $MgCl_2$, 0.2µM of each dNTP, 0.26µM of primers GP-E6-3F and GP-E6-5B, 0.75U of GoTaq DNA Polymerase (PROMEGA Corporation, Madison, USA) and 250ng of DNA. The conditions for the amplification of a 630-bp product were an initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min. The last cycle was followed by an elongation step at 72°C for 10 min. PCRs for L1 gene were performed in a final volume of 30µl containing 1X colorless reaction buffer, 2.5mM $MgCl_2$, 200µM of each dNTP, 0.1µM of primers MY09 and MY11, 2 U of GoTaq DNA Polymerase (PROMEGA Corporation, Madison, USA) and 150 ng of DNA for the amplification of a 450-bp product. The amplification program was performed with an initial denaturation at 94°C

for 3 min followed by 40 cycles of 1-min denaturation at 94°C, 1-min annealing at 55°C, and 1-min elongation at 72°C. The final extension step was done at 72°C for 10 min.

Genes E6 and L1 from samples infected with HPV16 (n=16) and HPV58 (n=12) were amplified, sequenced in Functional Biosciences (http://functionalbio.com/web/), and nucleotide sequences were concatenated. Each gene nucleotide sequence was aligned independently with references variants of HPV16 and HPV58 obtained from the NCBI GenBank using MEGA software version 6.0. The E6 and L1 genes independent alignments were concatenated manually for each strain. The phylogenetic trees, with a bootstrap analysis for 1000 replicates, were constructed by the maximum-likelihood algorithm using the same software.

## RESULTS

A total of 167 fresh cervical and vaginal biopsies were included in the study. The patients´ ages ranged from 19 to 77 years old. The lesions were divided in three groups according to their histopathological results: 47 samples (28%) with CIN II (Cervical Intraepithelial Neoplasia type II), 62 samples (37%) with CIN III and 58 samples (35%) with either cervical, or vaginal cancer (in situ or invasive) (Table 1). Out the 167 samples, 144 (86%) were positive for at least one of the HPV types screened; 35 out of 47 (74%) CIN II lesions, 55 of 62 (89%) CIN III lesions and 54 of 58 (93%) cancerous lesions were positive for HPV infection. From the total of samples analyzed, 59% were infected with only one HPV type and 27% had multiple infections with two or three types and 14% were negative for HPV DNA (data not shown). All co-infections had at least one high-risk HPV type, however, seven mono-infected samples had only low-risk types. HPV6 or HPV11 were detected in five samples showing CIN II and CIN III; HPV42 was identified in one CIN II lesion, and HPV43 in a patient with CIN III. No low-risk genotypes were detected in cancer lesions.

The results of genotyping showed that 19 out of 21 HPV types tested were detected at least once. Overall, HPV16 and HPV58 were the most frequently detected in mono-infections (61%, and 44%, respectively) and in co-infections (44%, and 42%, respectively) (data not shown). HPV16 was the most frequent genotype in both pre-cancerous (CIN II and CIN III) and cancerous lesions, while HPV58 was detected in all grade lesions (Table 1), followed by HPV52, 6/11, 66 and 31 in decreasing frequency. Intriguingly, HPV18 was only detected in four samples (2%).

Nucleotide sequences from HPV16 amplicons grouped mostly into the European branch (Lineage A) (Figure 1), and only one (sample 88M) clustered with the Asian-American variants (Sub-lineages D2/D3). Among sequences with high similarity to European

variants (Table 2), 20M was identical to A1 sub-lineage, while 14 sequences grouped into A2 sub-lineage and sample 69M clustered within lineage A but had one non-synonymous substitution shared with lineages B, C and D.

Regarding HPV58 amplicons, 10 out of 12 sequences grouped into sub-lineage A2 (Figure 2) but sequence from sample LM82 showed one synonymous substitution in common with sublineage A3, and amplicon from sample LM51 had one synonymous substitution and four non-synonymous substitutions present in lineage C sequences. No samples aligned with sub-lineage A1, lineages B or D (Table 3).

## DISCUSSION

This study is the first report of HPV type distribution and assessment on HPV58 variants among Ecuadorian women diagnosed with pre-cancer and cancerous lesions contributing to the understanding of HPV epidemiology in Ecuador. Two types HPV16 (61% in mono-infections and 44% in co-infections) and HPV58 (44% and 42%, respectively) were associated to pre-cancerous and cancerous lesions from Ecuadorian women attending to a major hospital in Quito. It was surprising to find high percentage of pre-cancerous and cancerous lesions associated to HPV58, although similar results have been described in Asia [Huang et al. 1997; C.-A. Chen et al. 2006], México [González-Losa et al. 2004; Piña-Sánchez et al. 2006], Nicaragua [Hindryckx 2006] and Brazil [Fernandes et al. 2013]. On the other hand, it was also unexpected to find very little (2.4%) of HPV18 associated to pre-cancerous and cancerous lesions; however, similar results have been described previously in Ecuador [Tornesello et al. 2008], Nicaragua [Hindryckx 2006] and Mexico [Piña-Sánchez et al. 2006]. This low frequency of HPV18 found in this study may be because this HPV type seems to be uncommon in South America (only 5% of HPV infections) as opposed to 11% in North America and 8% in Europe [Clifford 2005]. HPV18 has been detected in 5% of HPV positive samples in Ecuadorian women (data not shown). Some authors have proposed that complex interactions between HPV types and HLA haplotypes may explain the differences in circulating HPVs in different regions of the world [Clifford 2005].

In addition, a 27% of samples were infected with two or three HPV types. HPV6/11 were detected in cancer but always in co-infections with at least one HR HPV, which is in accordance to previously published results; no clear association between specific HPV combinations was observed as previously described in other reports [zur Hausen 2002; Thomas et al. 2000]. Seven pre-cancerous lesions (but no cancerous lesions) were

associated to infections with low risk HPV types, this finding also concurs with previous reports [zur Hausen 2002].

Nucleotide sequences of HPV16 variants clustered mainly with the European linage, most of them belonged to A2 sub-lineage (German branch), one sequence clustered with A1 sub-lineage (European lineage); and one sequence seemed to belong to the D2 sub-lineage, known as Asian-American 2 branch (figure 1). Some reports from other Latin-Americans countries have also described that most HPV16 variants are European [Schiffman et al. 2010; Villa et al. 2000; Sichero et al. 2007; Hildesheim et al. 2001; Picconi et al. 2003]. However, one study from Mexico found that 23.8% HPV16 variants belonged to European lineages and 23.2% to Asian-American sub-lineages [Berumen et al. 2001]. This is relevant because Non-European HPV16 sublineages (especially the Asian-American) tend to be more persistent [Villa et al. 2000], more aggressive and more carcinogenic than European counterparts [Burk, Harari, and Chen 2013; Z. Chen et al. 2011; Berumen et al. 2001; Sichero et al. 2007; Hildesheim et al. 2001]. Greater carcinogenicity may be associated to more efficient viral replication (higher copy number per cell), and greater p53 degradation by E6 protein [Berumen et al. 2001].

The phylogenetic analysis of Ecuadorian HPV58 sequences showed that most variants belonged to the A2 sub-lineages which may be the oldest HPV58 sub-lineage and from which the other lineages emerged [P. K. S. Chan et al. 2011]. One amplicon sequence clustered with sub-lineage A3; two substitutions found in the latter sequence have been associated previously to higher risk of cancer: E7 T20I causes greater affinity to the RB protein and E7 G35S creates an additional phosphorylation site which may increase oncogenic activity [Paul K. S. Chan et al. 2002].

Assessment of the oncogenic HPV types affecting Ecuadorian women is vital to analyze the possible impact of the vaccination program in our population. Since the bivalent HPV vaccine was introduced in Ecuador early 2014 [MSP 2014a], even though both vaccines, bivalent and tetravalent, have been available for more than 4 years; our results may advised less efficacy on reducing cancer in Ecuadorian women. The results presented here may suggest that this vaccine will be less efficacious in Ecuador than in other countries were HPV16 and HPV18 are the main causes of cervical cancer. Additionally it would be important to monitor mutations in capsid and oncogenic proteins for they will inform about predicted oncogenicity and vaccination success [Berumen et al. 2001]. The introduction of a nona-valent vaccine may reduce the combined incidence of high grade lesions in cervical/vulvar/vaginal disease (caused by genotypes 6, 11, 16, 18, 31, 33, 45, 52 and 58) in 96.7% [Eisele et al. 2013].

As the number of some variants is very small, it is hard to compare each variant category and it does not allow additional analysis. Nevertheless, as the study focuses only on pre-cancer and cancer, its results are of great interest to understand better the epidemiology and frequency of HPVs present in Ecuador. Moreover, the samples analyzed came from an nationwide oncologic referral center which may allow us to infer that similar results will be found in patients form different regions of Ecuador. It is also noteworthy that this study probably is the first report of HPV58 molecular variants done outside Asia. Pathological characteristics of HPV 58 variants and its associations with cancer needs further studies.

## Acknowledgements

This work was funded by Universidad San Francisco de Quito. The authors thank Hybribio Limited (Hong Kong, China) for their kindly support by providing "21 HPV GenoArray Diagnostic Kits" for HPV genotyping. The authors also thank Kristin Tomey for her valuable comments and her edition to the text.

## Financial support

This work was financed by Universidad San Francisco de Quito.

## Disclaimer

The authors declare no conflict of interest.

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Table 1. Most frequent HPV types (mono- and co-infections) found in precancerous and cancerous lesions in Ecuadorian women attending a large hospital in Quito. CIN: Cervical Intraepithelial Neoplasia. * Cancer biopsies include cervical and vaginal samples (n=167). Some samples had more than one HPV type

| | n | HPV+ (%) | HPV16 (%) | HPV58 (%) | HPV52 (%) | Others (%) |
|---|---|---|---|---|---|---|
| **CIN II** | 47 | 35 (74) | 13 (37) | 7 (20) | 5 (14) | 23 (66) |
| **CIN III** | 62 | 55 (89) | 24 (44) | 20 (36) | 5 (9) | 27 (49) |
| **Cancer *** | 58 | 54 (93) | 24 (44) | 17 (31) | 7 (13) | 26 (48) |
| **Total** | **167** | **144 (86)** | **61 (42)** | **44 (31)** | **17 (12)** | **76 (53)** |

Table 2. Variable sites alignment of E6 and L1 genes from HPV16 variants obtained in this study. Histopathology diagnosis of the sequences reported here are denoted as CIN (cervical intraepithelial neoplasia) II, CIN III and cancer. The rest of the sequences were obtained from GenBank. Representative genomes for HPV16 lineages (termed A, B, C and D) and sublineages (termed A1-A4/B1-B2/D1-D3) were used as variant references [Burk, Harari, and Chen 2013]. Regional variants are indicated as: E, European variant; As, East Asian; Afr, African; NA, North American; AA, Asian-American. The sequences order is based on the topology of figure 1.

| HPV16 Variants | E6 gene | | | | | | | | | | | | | | | | L1 gene | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 43 | 69 | 91 | 92 | 103 | 105 | 138 | 143 | 236 | 246 | 249 | 295 | 310 | 363 | 393 | 492 | 625 | 628 | 694 | 704 | 730 | 812 | 835 | 863 | 871 | 874 | 979 | 1003 | 1030 |
| HPV16. A1. K02718. E | A | T | A | G | C | G | T | T | A | T | A | C | T | A | G | A | T | C | T | A | G | A | C | C | T | C | C | G | G |
| **20M Cancer** | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| HPV16. A2. AF536179. E (German) | . | . | G | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | C | . | . | A | . |
| **68M Cancer** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | G | A | . | . | . | . | . | . | . | . | . | . | . |
| **84M CIN III** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **70M CIN III** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM160 CIN III** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM143 CIN II** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM112 CIN III** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM62 Cancer** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM46 Cancer** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM33 CIN III** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM19 CIN III** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM21 Cancer** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM42 Cancer** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **LM55 Cancer** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| **69M Cancer** | . | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . |
| HPV16. A3. HQ644236. E | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . |
| HPV16. A4. AF534061 E (As) | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| HPV16. B1. AF536180. Afr1a | C | . | . | C | G | T | . | . | . | A | G | T | . | . | . | . | . | . | . | . | A | . | . | T | . | . | T | A | . |
| HPV16. B2. HQ644298. Afr1b | . | . | G | . | G | T | . | . | . | A | G | T | . | . | . | . | . | . | A | . | A | . | . | T | . | . | T | A | . |
| HPV16. C. AF472509. Afr2a | . | C | . | T | G | T | . | . | . | A | G | T | . | G | . | . | . | . | . | C | A | . | . | T | . | T | T | A | . |
| HPV16. D1. HQ644257. NA1 | . | . | . | . | . | T | . | . | . | A | G | T | G | . | . | . | . | . | . | C | A | . | . | T | . | T | T | A | T |
| HPV16. D2. AY686579. AA2 | . | . | . | . | . | T | . | . | . | A | G | T | G | . | . | G | . | . | . | C | A | . | . | T | C | T | T | A | . |
| **88M Cancer** | . | . | . | . | . | T | . | G | . | A | G | T | G | . | . | G | . | . | . | C | A | . | . | T | C | T | T | A | . |
| HPV16. D3. AF402678. AA1 | . | . | . | . | . | T | . | . | . | A | G | T | G | . | A | G | . | . | . | C | A | T | . | T | . | T | T | A | . |

Table 3. Variable sites alignment of E6 and L1 genes from HPV18 variants obtained in this study. Histopathology diagnosis of the sequences reported here are denoted as CIN (cervical intraepithelial neoplasia) II, CIN III and cancer. The rest of the sequences were obtained from GenBank. Representative genomes for HPV58 lineages (termed A, B, C and D) and sub-lineages (termed A1-A3/B1-B2/D1-D2) were used as variant references [Z. Chen et al. 2011]. The order of the sequences is based on the topology shown in figure 2.

| HPV58 Variants | E6 gene | | | | | | | | | | | | | | | | | | L1 gene | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 21 | 34 | 73 | 80 | 98 | 124 | 130 | 140 | 182 | 183 | 244 | 304 | 325 | 329 | 335 | 337 | 524 | | 575 | 578 | 583 | 587 | 592 | 606 | 641 | 693 | 717 | 722 | 723 | 776 | 905 | 911 | |
| HPV58. A1. LZcc86 | A | C | A | T | G | C | G | G | G | T | C | C | A | T | A | T | A | | T | A | C | G | G | G | G | A | G | C | A | A | A | A | |
| HPV58. A1. Sc101 | . | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | | . | . | . | . | . | . | A | . | . | . | . | . | . | . | |
| HPV58. A2. Qv15606 | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | | . | . | . | . | . | . | A | . | . | . | . | . | . | . | |
| HPV58. A2. Rw791 | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | | . | . | . | . | . | . | A | . | . | . | . | . | . | . | |
| **LM44 CIN III** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **LM94 CIN III** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **LM11 CIN II** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **65M Cancer** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **64M Cancer** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **44M Cancer** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **42M CIN II** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **35M CIN III** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **12M Cancer** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| **LM126 CIN III** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | . | . | . | . | . | . | **A** | . | . | . | . | . | . | . | |
| HPV58. A3. Qv00961 | . | . | G | . | . | . | . | . | . | . | T | . | . | . | . | . | . | | . | G | . | . | . | . | A | . | . | A | . | G | . | . | |
| HPV58. A3. AS347 | T | . | . | . | . | . | . | . | . | . | T | . | . | . | . | . | . | | . | . | . | . | . | . | A | . | . | A | . | . | . | . | |
| **LM82 Cancer** | . | . | . | . | . | . | . | . | . | . | **T** | . | . | . | . | . | . | | **G** | . | . | . | . | . | **A** | . | . | **A** | . | . | . | . | |
| HPV58. B1. Z023 | . | . | . | . | . | . | . | C | . | . | T | A | C | . | . | . | . | | . | . | . | . | . | . | A | G | A | A | G | . | . | . | |
| HPV58. B1. BF134 | . | . | . | . | . | . | . | C | . | . | T | A | . | . | . | . | . | | . | . | . | . | . | . | A | G | A | A | G | . | . | . | |
| HPV58. B2. Rw937 | . | T | . | . | . | . | . | . | . | . | T | A | . | . | . | . | . | | . | . | . | . | . | . | A | G | A | A | G | . | . | . | |
| HPV58. B2. Rw754 | . | T | . | . | . | . | . | . | . | . | T | A | . | . | . | . | . | | . | . | . | . | . | . | A | G | A | A | G | . | . | . | |
| HPV58. C. Qv34982 | . | . | . | . | . | T | . | . | . | . | T | A | . | . | . | . | . | | . | . | . | A | A | A | A | G | A | A | G | . | . | G | |
| HPV58. C. Rw792 | . | . | . | . | A | T | . | . | C | . | T | A | . | . | G | . | . | | . | . | . | A | A | A | A | G | A | A | G | . | . | G | |
| **LM51 CIN II** | . | . | . | . | . | **T** | . | . | . | . | **T** | **A** | . | . | . | . | . | | . | . | . | **A** | **A** | **A** | **A** | **G** | **A** | **A** | **G** | . | . | **G** | |
| HPV58. D1. Qv03858 | . | . | . | C | . | . | A | . | A | . | T | . | . | . | G | . | G | | . | . | . | . | . | . | A | . | A | A | G | . | G | G | |
| HPV58. D1. Qv04732 | . | . | . | . | . | . | A | . | A | . | T | . | . | . | G | . | G | | . | . | . | . | . | . | A | . | A | A | G | . | G | G | |
| HPV58. D2. Rw841 | . | . | . | . | . | . | . | . | A | C | T | . | . | C | G | G | . | | . | . | A | A | . | . | A | G | A | A | G | . | G | G | |
| HPV58. D2. Rw63 | . | . | . | . | . | . | . | . | A | C | T | . | . | C | G | G | . | | . | . | A | . | . | . | A | G | A | A | G | . | G | G | |

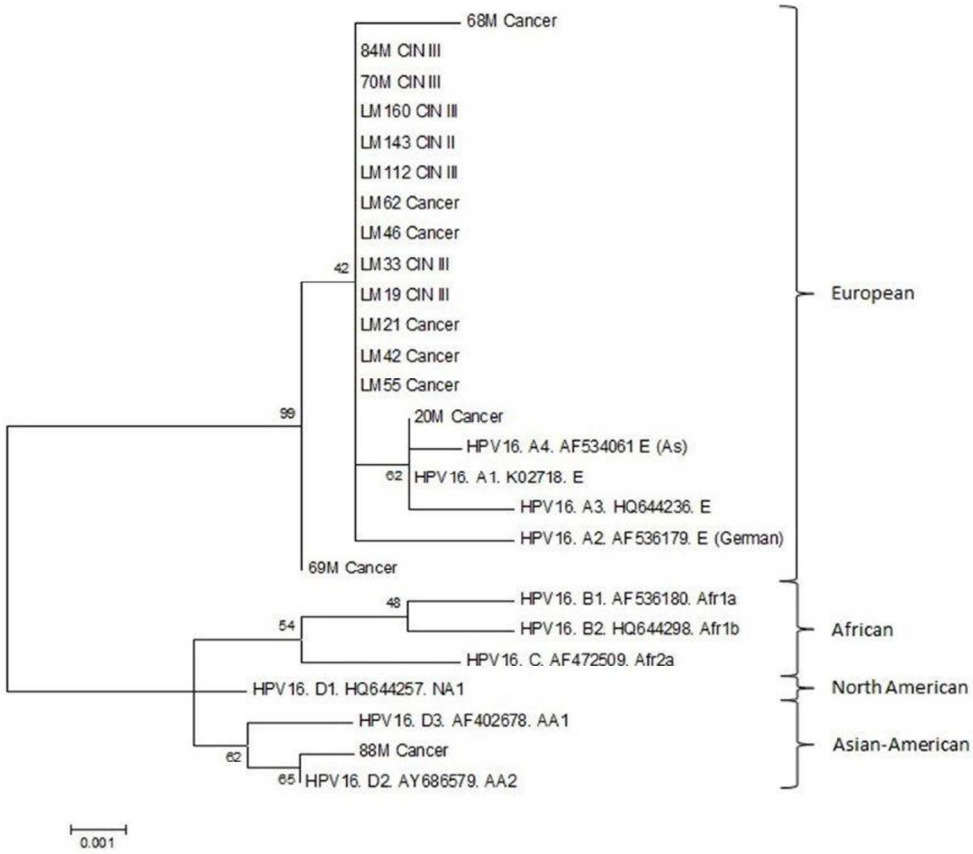


Figure 1. Maximum-likelihood tree using nucleotide sequences of E6 and L1 genes from HPV16 variants obtained in this study. Histopathology diagnosis are denoted as CIN (cervical intraepithelial neoplasia) II, CIN III and cancer. The rest of the sequences were obtained from GenBank. Numbers indicate bootstrap values from 1000 pseudo-replicates. Representative genomes for HPV16 lineages (termed A, B, C and D) and sub-lineages (termed A1-A4/B1-B2/D1-D3) were used as variant references [Burk, Harari, and Chen 2013]. Regional variants are indicated as: E, European variant; As, East Asian; Afr, African; NA, North American; AA, Asian-American.
101x87mm (600 x 600 DPI)

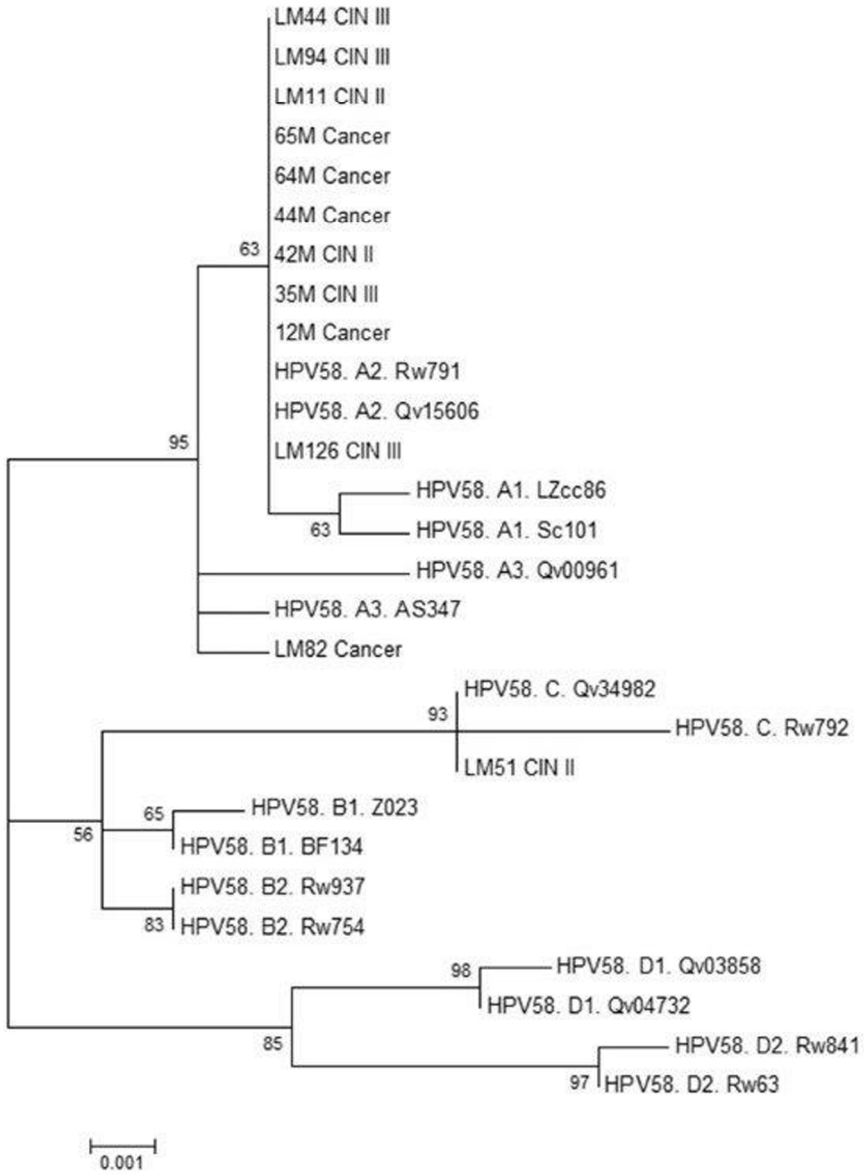


Figure 2. Maximum-likelihood tree using nucleotide sequences of E6 and L1 genes from HPV18 variants obtained in this study. Histopathology diagnosis of the sequences reported here are denoted as CIN (cervical intraepithelial neoplasia) II, CIN III and cancer. The rest of the sequences were obtained from GenBank. Numbers indicate bootstrap values from 1000 pseudo-replicates. Representative genomes for HPV58 lineages (termed A, B, C and D) and sub-lineages (termed A1-A3/B1-B2/D1-D2) were used as variant references [Z. Chen et al. 2011].
101x137mm (600 x 600 DPI)