E6/E7 Variants of Human Papillomavirus 16 Associated with Cervical Carcinoma in Women in Southern Mexico

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Abstract: Persistent infection with the human papillomavirus 16 (HPV 16) is the cause of half of all cervical carcinomas (CC) cases. Moreover, mutations in the oncoproteins E6 and E7 are associated with CC. In this study, E6/E7 variants circulating in southern Mexico and their association with CC and its precursor lesions were evaluated. In total, 190 DNA samples were obtained from scrapes and cervical biopsies of women with HPV 16 out of which 61 are from patients with CC, 6 from patients with high-grade squamous intraepithelial lesions (HSIL), 68 from patients with low-grade squamous intraepithelial lesions (LSIL), and 55 from patients without intraepithelial lesions. For all E7 variants found, the E7-C732/C789/G795 variant (with three silent mutations) was associated with the highest risk of CC (odd ratio (OR) = 3.79, 95% confidence interval (CI) = 1.46–9.85). The analysis of E6/E7 bicistron conferred to AA-a*E7-C732/C789/G795 variants revealed the greatest increased risk of CC (OR = 110, 95% CI = 6.04–2001.3), followed by AA-c*E7-C732/C789/G795 and A176/G350*E7-p. These results highlight the importance of analyzing the combinations of E6/E7 variants in HPV 16 infection and suggest that AA-a*E7-C732/C789/G795, AA-c*E7-C732/C789/G795, and A176/G350*E7-p can be useful markers for predicting CC development.

Keywords: human papillomavirus 16; E6; E7; cervical cancer; squamous intraepithelial lesion; E7 non-synonymous mutation

1. Introduction

Persistent infection with high-risk human papillomavirus (HR-HPV) is the main cause of cervical carcinoma (CC) and its precursor lesions [1]. Among the HR-HPV types, HPV 16 causes more than half of CC cases worldwide [2,3].

The oncogenicity of HPV 16 is because of the oncoproteins E6 and E7, which promotes cell transformation and immortalization; increase cell proliferation; invasion and migration; and supports immune evasion. E6 induces p53 degradation and prevents cell growth arrest and apoptosis. E7 binds to retinoblastoma proteins p107 and p130 and causes immortalization and deregulation of cell proliferation and differentiation. Hence, these oncoproteins act together to promote cervical carcinogenesis [4–6].

E6 and E7 genes are located in tandem at the beginning of the HPV 16 genome at nucleotides 104–559 and 562–858, respectively [7], and are initially transcribed as a single bicistronic HPV 16 E6/E7 pre-mRNA. Mature mRNA from the unspliced HPV 16 E6/E7...
bicistron encodes both oncoproteins, spliced transcripts, and E6*I/E7 and E6*II/E7, which are short forms of E6 with intact E7 [8,9].

HPV 16 belongs to the genus Alphapapillomavirus, species group A9 [10]. It has intratypic variants associated with premalignant lesion progression and CC development [11–14]. HPV 16 variants have been classified into four lineages: A (European-Asian, EAS), B (African 1, AF1), C (African 2, AF2), and D (North-American/Asian-American, NA/AA). Furthermore, nine sublineages have been described: A1, A2, A3 (European, E), A4 (Asian, As), B1 (Afr1a), B2 (Afr1b), D1 (North-American, NA), D2 (Asian-American, AA1), and D3 (Asian-American, AA2) [12,15]. Most infections worldwide are caused by sublineages A1, A2, and A3 [15,16]. However, sublineage D2 is closely associated with the risk of CC [14].

In Mexico, several E6 variants of HPV 16 have been characterized. In Mexico City, the variants E-G131 (A1) E-G350, E-C188, E-A176 (A2), NA1 (D1), AA-a, and AA-c (D2) were reported and among them the AA variants are more oncogenic than the E variants [17]. In other studies, only the E-G350 (A2) variant was reported [18,19]. In southeastern Mexico, E-Prototype (A1), E-G350 (A2), A12 (C), and AA (D2) were reported in samples from women with CC [20]. Moreover, in southern Mexico, we previously detected 27 E6 variants of HPV 16 and among them the most frequent and most closely associated with the development of CC were E-G350, E-C188/G350, E-176/G350 (A2), and AA-a and AA-c (D2) [21]. These HPV 16 E6 variants alter the expression of genes involved in proliferation, differentiation, apoptosis, cell cycle, adhesion, angiogenesis, transcription, and protein translation in C33-A cells and are consistent with the oncogenic potential of HPV 16 variants [22].

To date, few studies have focused on the intratypical variants of E7 as compared with those on E6 variants. Nevertheless, several studies demonstrated that non-synonymous mutations in E7 gene can cause amino acid changes. In particular, the variants A647G (NS29) and C749T (S63F) are associated with the progression of premalignant lesions into CC [23–25]. However, in a large study, E7 conservation was found to be significantly associated with CC, whereas E7 mutations were associated with reduced carcinogenicity of HPV 16 [13].

Taken together, a deeper understanding of the regional variants of HPV 16 is needed owing to their important epidemiological, preventive, and therapeutic implications for CC. Hence, the present study had aimed to perform the following: (1) to characterize the E7 genetic variations (since E6 variants circulating in southern Mexico have already been reported); (2) to determine the combinations of E6 and E7 variants in the HPV 16 E6/E7 bicistron in women from southern Mexico; and (3) evaluate the associations between the observed variants alone and in combination with the development of CC and precursor lesions.

2. Results

A total of 190 HPV 16 positive women (aged between 17 and 97 years) were included in the study. All participants were residents from the seven regions of the Guerrero state in southern Mexico. The average age of women with CC, high-grade squamous intraepithelial lesions (HSIL), low-grade squamous intraepithelial lesions (LSIL), and without intraepithelial lesions was 30.5, 36.3, 30.2, and 35.9 years, respectively. The clinical and demographic characteristics of the patients are presented in Table 1.
Table 1. Clinical and demographic characteristics of patients included in the study.

|                      | Non IL | LSIL | HSIL | CC   | Total |
|----------------------|--------|------|------|------|-------|
|                      | n      | %    | n    | %    | n     | %    |
| Mean age             | 35.9   | 30.2 | 36.3 | 50.5 | 38.6  | <0.005|
|                      | 32     | 40   | 58.2 | 1    | 50.00 | 4.2  |
| Age                  |        |      |      |      |       |
|                      | 25     | 12   | 16   | 9    | 9     | <0.005|
|                      | 40     | 19   | 27.94 | 16   | 4.2  |
|                      | 7      | 1    | 10.29 | 33.33 | 31   | 37.19 |
|                      | 2      | 2    | 2.94 | 0    | 26    |
|                      |        |      |      |      |       |
| Age at menarche      |        |      |      |      | <0.363|
|                      | 32     | 23   | 41.82 | 37   | 23    |
|                      | 43     | 21   | 41.82 | 37   | 23    |
|                      | 50     | 30.88 | 3   | 16.67 | 13    |
|                      | 50.82  | 37.70 | 3    | 50.00 | 11    |
|                      |        |      |      |      |       |
| Age at menopause     |        |      |      |      | <0.005|
|                      | 3      | 2     | 2.94 | 0    | 3     |
|                      |        |      |      |      |       |
| Age at first sexual intercourse | | | | | <0.005|
|                      | 5      | 7     | 7.27 | 0    | 2     |
|                      |        |      |      |      |       |
| Geographical region of Guerrero | | | | | <0.005|
| Costa Chica          | 0      | 0     | 1.47 | 0    | 0     |
|                      |        |      |      |      |       |
| Costa Grande         | 0      | 0     | 0    | 0    | 0     |
|                      |        |      |      |      |       |
| La Montaña           | 1      | 5     | 5.45 | 6    | 6.66  |
|                      |        |      |      |      |       |
| Tierra Caliente      | 1      | 1.82  | 2    | 0    | 0     |
|                      |        |      |      |      |       |
| Acapulco             | 5      | 9.09  | 6    | 8.82 | 0     |
|                      |        |      |      |      |       |
| Zona Centro          | 41     | 74.55 | 51   | 75.00 | 6   |
|                      |        |      |      |      |       |
| Zona Norte           | 2      | 3.64  | 3    | 4.41 | 0     |
|                      |        |      |      |      |       |
| Did not answer       | 3      | 5.45  | 3    | 1.47 | 0     |
|                      |        |      |      |      |       |
| Total                | 55     | 100   | 68   | 100  | 61    | 100  |

Abbreviations: Non IL, non intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CC, cervical carcinoma.

2.1. E7 Variants of HPV 16

E7 variants in HPV 16 identified in women in southern Mexico with CC, HSIL, LSIL, and non-intraepithelial lesions were analyzed. Eight E7 mutations were found and comprises two missense mutations (A647G and C712A) and six synonymous mutations (G666A, T678C, T732C, C765T, T789C, and T795G). The E7 prototype was detected most frequently (74.74%), followed by the E7-C732/C789/G795 variant (20.53%) with three silent mutations and a genotype that was associated with the highest risk of CC (odd ratio (OR) = 3.79, 95% confidence interval (CI) = 1.46–9.85). Other variants with synonymous mutations were detected, including E7-A666 (2.11%), E7-C678 (0.53%), and E7-T765 (0.53%). Variants with non-synonymous mutations were found in 2.11% (A712) and 0.53% (G647) of the samples (Table 2).
Table 2. Frequency of HPV 16 E7 genetic variants and their association with cervical carcinoma and precursor lesions in women from southern Mexico.

| E7 region | Non IL | LSIL | HSIL | CC | Total |
|-----------|--------|------|------|----|-------|
| Variants  | n %    | n %  | n %  | n %|       |
| E7-prototype | 48 | 87.27 | 53 | 77.94 | - |
| E7-C789/G795 | 7 | 12.72 | 13.76 | 1.03 | 0.950 | 3 | 50.0 | 1.06 | 0.55 | 21 | 34.42 | 0.006 | 39 | 20.55 |
| E7-A666 | 0 | 0.00 | 2 | 2.94 | - | 0 | 0 | 0 | 0 | 2 | 3.28 | - | 4 | 2.11 |
| E7-A712 | 0 | 0.00 | 2 | 2.94 | - | 0 | 0 | 0 | 0 | 0 | 0 | - | - | 2 | 1.05 |
| E7-T765 | 0 | 0.00 | 1 | 1.47 | - | 0 | 0 | 0 | 0 | 0 | 0 | - | - | 1 | 0.53 |
| E7-G647/C789/G795 | 0 | 0.00 | 1 | 1.47 | - | 0 | 0 | 0 | 0 | 0 | 0 | - | - | 1 | 0.53 |

Note: Capital letters indicate the mutations that generate amino acid change and lowercase letters indicate mutation without amino acid change. Abbreviations: Non IL, non intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CC, cervical carcinoma; CI, confidence interval; OR, odd ratio. For the amino acid change prediction was made on HPV 16 reference, sequence NC 1526.3, indicates reference category (E7-Prototype).
2.2. E6/E7 HPV 16 Bicistronic Variants of HPV 16

E6 and E7 variant combinations in the HPV E6/E7 bicistron were analyzed and grouped by sublineage. Four of the nine HPV 16 sublineages were present in the studied population: A1 (12.63%), A2 (66.84%), C1 (0.53%), and D2 (20%).

Mutations in the E6 and E7 genes of HPV 16 were identified in the same patient, and 34 bicistron combinations were detected. The most frequent combination of E6/E7 variants in HPV 16 was the E-G350*E7-prototype (36.8%), which belongs to the A2 sublineage, and the second most frequent combination was AA-a*E7-C732/C789/G795 (10%), which belongs to the D2 sublineage. Only 9.5% of the samples had a combination of E6 and E7 prototypes (Table 3).

In order to determine the effect of the combination of E6 and E7 variants in women infected with HPV 16, the risk of CC and its precursor lesions was analyzed. The risk of CC was highest in individuals harboring AA-a*E7-C732/C789/G795 (OR = 110, 95% CI = 6.04–2001.3), AA-c*E7-C732/C789/G795 (OR = 35, 95% CI = 2.63–465.37), and E-A176/G350*E7-prototype (OR = 23.33, 95% CI = 1.99–273.3). The oncogenic risk of the most common bicistronic variant, the E-G350*E7-prototype, was not statistically significant. The risk of developing CC precursor lesions was not significant for any of the E6/E7 combinations. The E-prototype*E7-prototype bicistron was used as a reference (Table 3).

3. Discussion

The aim of this study was to improve our understanding of HPV 16 variants circulating in southern Mexico owing to its epidemiological, preventive, and therapeutic implications for CC. We have previously reported that the most frequent E6 variants of HPV 16 and those associated with a high risk of developing CC were the European A1 and A2 sublineages (E-G350, E-prototype, E-A176/G350, and E-C188/G350) and Asian-American D2 (AA-a and AA-c) lineages [21]. This is the first study of E7 variants in HPV 16 in Mexico involving the analyses of variant combinations in the HPV E6/E7 bicistron in cervical samples as well as their association with the development of CC and its precursor lesions.

Herein, fewer variants were detected in E7 than in E6 of HPV 16, which in agreement with a previous report [21]. In addition to the numerical difference, only two variants identified—A712 (H51N) and G647 (N29S)—altered the E7 amino acid sequence and were detected at a very low frequency in southern Mexico. However, all the E6 variants showed amino acid changes [21].

Mutations in E7 herein identified were mostly synonymous (G666A, T678C, T732C, C765T, T789C, and T795G). Although these mutations do not change the amino acid residues of the oncoprotein, they are present at a high frequency in CC as is the case with the E7-C732/C789/G795 variant, which is associated with a 3.79 times higher risk of developing this pathology than compared with the E7-prototype. Moreover, E7-C732/C789/G795 was the second most frequent variant after the E7-prototype. What is noteworthy is that these three silent mutations were previously reported in Panama, United States, Suriname, Thailand, and India where they were found in patients with CC [26–30]. However, these reports did not evaluate the potential associations between the C732/C789/G795 variants in E7 of HPV 16 and the development of CC. The E7 variant that was previously reported to be associated with CC was E7-G647 [24,31], while in this study it was E7-C732/C789/G795. Although synonymous mutations do not modify the amino acid sequence of the oncoprotein, they may affect the structure, splicing, or stability of the RNA [32,33], thus contributing to oncogenicity; however, further studies are required to evaluate the precise effects. Taken together, these data emphasize the importance of analyzing local E7 variants and their association with the development of CC.

The distribution of HPV 16 E7 variants differs worldwide. In the American continent, the most reported variant is E7-C732/C789/G795; in Africa, it is E7-C789/G795; in Asia, they are E7-G647/C846, E7-G647, E7-C646, E7-A666, and E7-C732/C789/G795; and in Europe they are E7-C732/T749/G795, E7-G822, and E7-C789/G795 (Table 4). In this study, the most frequent E7 variant was E7-C732/C789/G795, which concurs with what has been reported in other countries in the American continent.
### Table 3. Frequency of combinations of E6 and E7 variants in the HPV 16 E6/E7 bicistron and its associations with cervical carcinoma and precursor lesions.

| Sublineage | E6 Variants | E7 Variants | Non IL | LSIL | HSIL | CC | Total |
|------------|-------------|-------------|--------|------|------|----|-------|
|            | n | % | n | % | OR (95% IC) | p | n | % | OR (95% IC) | p | n | % | OR (95% IC) | p | n | % | OR (95% IC) | p | n | % | OR (95% IC) | p |
| A1         | 13 | 23.6 | 7 | 10.3 | 1 | 16.7 | 3 | 4.9 | 24 | 12.6 |
| E-prototype | E-prototype | E-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | E7-prototype | 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Table 4. Most frequent HPV 16 E7 variants reported around the world.

| E7 HPV16 Variant          | E7-prototype | Country | Reference |
|--------------------------|--------------|---------|-----------|
| Total CC Non-IL/SIL Total CC | Total CC N N % N % |         |
| Africa                    |              |         |           |
| E7-C789/G795             | 21 21 0 9 43 9 43 3 14 3 14 | Tanzania | Eschle et al., 1992 [34] |
| E7-C789/G795             | 5 3 2 5 100 3 100 0 0 0 0 | Uganda | Buonaguro et al., 2000 [35] |
| E7-C789/G795             | 13 13 0 13 100 13 100 0 0 0 0 | Congo | Bouma et al., 2015 [36] |
| America                  |              |         |           |
| E7-C732/C789/G795        | 190 61 129 39 21 21 34 142 75 38 62 | Mexico | This study |
| E7-C732/C789/G795        | 100 0 100 16 16 0 0 72 72 0 0 | EU(Texas) | Swan et al., 2005 [28] |
| E7-C732/C789/G795        | 25 25 0 3 12 3 12 15 60 15 60 | Suriname | De Boer et al., 2004 [27] |
| E7-C732/C789/G795        | 15 ND ND 3 20 ND ND 8 53 ND ND | Panama, EU | Ikenogle et al., 1991 [26] |
| E7-A712                  | 12 0 12 2 17 0 0 6 50 0 0 | Uruguay | Ramas et al., 2018 [37] |
| E7-C732/C789/G795        | 11 11 0 6 55 6 55 3 27 3 27 | Barbados | Smits et al., 1994 [38] |
| E7-C732                  | 52 ND ND 7 13 ND ND ND ND | Costa Rica | Saha et al., 2010 [39] |
| E7-C789                  | 52 ND ND 8 15 ND ND ND ND | Costa Rica | Saha et al., 2010 [39] |
| E7-G795                  | 52 ND ND 8 15 ND ND ND ND | Costa Rica | Saha et al., 2010 [39] |
| Asia                     |              |         |           |
| E7-G647/C846             | 292 0 292 125 43 0 0 125 43 0 0 | China | Zhang et al., 2015 [40] |
| E7-G647/C846             | 167 53 114 126 75 37 70 7 4 2 4 | China | Ding et al., 2010 [41] |
| E7-G647/C846             | 98 0 98 63 64 0 0 14 14 0 0 | China | Zhao et al., 2020 [42] |
| E7-G647/C846             | 97 0 97 62 64 0 0 13 13 0 0 | China | Cao et al., 2016 [43] |
| E7-G647/C846             | 76 76 0 26 34 26 34 15 20 15 20 | China | Sun et al., 2012 [44] |
| E7-G647/C846             | 47 47 0 29 62 29 62 0 0 0 0 | China | Wu et al., 2006 [23] |
| E7-G647/C846             | 58 38 20 36 62 26 68 ND ND | China | Yang et al., 2014 [45] |
| E7-G647/C846             | 157 43 114 128 82 41 95 4 3 ND ND | Taiwan | Chang et al., 2013 [51] |
| E7-G647/C846             | 15 9 6 6 40 5 56 3 20 2 22 | Japan | Fujinaga et al., 1994 [46] |
| E7-G647/C846             | 31 0 31 18 58 0 0 2 10 0 0 | Japan | Ishizaki et al., 2013 [47] |
| E7-G647/C846             | 24 0 24 5 21 0 0 18 75 0 0 | Philippines | Ishizaki et al., 2013 [47] |
| E7-G647/C846             | 24 0 24 20 83 0 0 0 0 0 0 | Vietnam | Ishizaki et al., 2013 [47] |
| E7-G647/C846             | 9 9 0 3 33 3 33 3 33 3 33 | Thailand | Chansaenroj et al., 2012 [29] |
| E7-C732/C789/G795        | 9 9 0 3 33 3 33 3 33 3 33 | Thailand | Chansaenroj et al., 2012 [29] |
Table 4. Cont.

| Variant        | E7 HPV16 Variant | E7-prototype |
|----------------|------------------|--------------|
|                | Total CC | Non-IL/SIL Total CC | Total CC | Country | Reference |
|                | N  | N  | N  | N  | %  | N  | N  | %  | N  | %  |
| **Asia**       |         |         |         |         |     |     |     |     |     |
| E7-G647        | 141    | 25    | 116   | 63    | 45  | 18  | 72  | 55  | 39  | 5  | 20  | Korea | Lee et al., 2011 [24] |
| E7-G647        | 133    | 31    | 102   | 118   | 89  | 55  | 41  | 15  | 11  | 2  | 7   | Korea | Park et al., 2016 [48] |
| E7-G647        | 56     | 0     | 56    | 36    | 64  | 0   | 0   | 7   | 13  | 0  | 0   | Korea | Choi et al., 2007 [49] |
| E7-G647        | 42     | 27    | 15    | 25    | 60  | 19  | 70  | 8   | 19  | 3  | 11  | Korea | Song et al., 1997 [50] |
| E7-G647        | 143    | 81    | 62    | 54    | 38  | 29  | 36  | ND  | ND  | ND | ND  | India | Radhakrishna Pillai et al., 2002 [51] |
| E7-G647        | 43     | 43    | 0     | 1     | 2   | 1   | 2   | 42  | 98  | 42  | 98  | China | He et al., 2016 [52] |
| E7-G647        | 31     | 0     | 31    | 24    | 77  | 0   | 0   | ND  | ND  | ND | 0   | China | Zhao et al., 2019 [53] |
| E7-G647        | 31     | 31    | 0     | 19    | 61  | 19  | 61  | 9   | 29  | 9  | 29  | Thailand | Vaeteewootacharn et al., 2003 [54] |
| E7-C646        | 100    | 0     | 100   | 21    | 21  | 0   | 0   | ND  | ND  | ND | 0   | China | Zhang et al., 2016 [55] |
| E7-A666        | 52     | 52    | 0     | 20    | 39  | 20  | 39  | 8   | 15  | 8  | 15  | China | Shang et al., 2011 [56] |
| E7-A666        | 22     | 22    | 0     | 15    | 68  | 15  | 68  | 2   | 9   | 2  | 9   | Indonesia | De Boer et al., 2004 [27] |
| E7-C789/G795   | 124    | 28    | 96    | 8     | 7   | 4   | 14  | 103 | 83  | 19  | 68  | Italy | Tornesello et al., 2004 [62] |
| E7-C732/C789/G795 | 110  | 94    | 16    | 12    | 11  | 11  | 12  | 95  | 86  | 80  | 85  | India | Mazumder Indra et al., 2011 [30] |

|                |         |         |         |         |     |     |     |     |     |
| **Europe**     |         |         |         |         |     |     |     |     |     |
| E7-C732/T749/G795 | 112  | 37    | 75    | 15    | 13  | 4   | 11  | 89  | 80  | 32  | 87  | Romania | Plesa et al., 2014 [58] |
| E7-G822        | 61     | 31    | 30    | 4     | 7   | 2   | 7   | 55  | 90  | 27  | 87  | Germany | Nindl et al., 1999 [59] |
| E7-G822        | 40     | 40    | 0     | 2     | 5   | 2   | 5   | 35  | 88  | 35  | 88  | Sweden | Hu et al., 2001 [60] |
| E7-G822        | 90     | 36    | 54    | 10    | 11  | 4   | 11  | 71  | 79  | 24  | 67  | Slovenia | Vrtačnik Bokal et al., 2010 [61] |
| E7-C789/G795   | 14     | 14    | 0     | 1     | 7   | 0   | 0   | 13  | 93  | 0   | 0   | Italy | Garbuglia et al., 2007 [63] |
| E7-C789/G795   | 27     | 27    | 0     | 3     | 11  | 3   | 11  | 21  | 78  | 21  | 78  | Netherlands | De Boer et al., 2004 [27] |
| E7-C732/C789/G795 | 29  | 29    | 1     | 3     | 0   | 0   | 27  | 93  | 0   | 0   | Italy | Cento et al., 2009 [64] |
| E7-T616        | 8      | 3     | 5     | 1     | 13  | 1   | 33  | 7   | 88  | 2  | 67  | Netherlands | Smits et al., 1994 [38] |
| -              | 6      | 6     | 0     | 0     | 0   | 0   | 0   | 6   | 100 | 6  | 100 | Germany | Eschle et al., 1992 [34] |
The complementary and synergistic effects of the HPV 16 oncoproteins E6 and E7 play a critical role in malignant transformation and cell immortalization [1]. There is extensive evidence that HPV 16 E6 and E7 mutations influence persistence, progression to precursor lesions, and the development of CC [12]. The differential oncogenic risk among variants of each oncoprotein has been studied; however, it is also necessary to analyze the oncogenic risk of combinations of E6/E7 variants.

One of the few studies on the oncogenic risk of the E6/E7 variant combination in HPV 16 focused on Korean women, in which the E6 T178G and E7 A647G (As*E7-G647) combination was the most prevalent and showed a significant association with the severity of cervical neoplasia [24]. In the present study, we found that individuals carrying the AA-a*E7-C732/C789/G795 combination possess the highest risk of CC (OR = 110), further suggesting that there may be a synergistic effect between the loci, as is evidenced by the lower risk for each locus individually (OR = 69.01 for AA-a of E6 [21] and 3.79 for E7-C732/C789/G795 in this study). The combinations of AA-c*E7-C732/C789/G795 and E-A176/G350*E7-prototype were also related to the risk of progression of precursor lesions to CC.

In conclusion, this is the first study of E7 variants of HPV 16 in Mexican women. Notably, E7-C732/C789/G795, a variant with three silent mutations, was associated with the risk of developing CC. These results highlight the importance of analyzing combinations of E6 and E7 variants in HPV 16 and suggest that AA-a*E7-C732/C789/G795, AA-c*E7-C732/C789/G795, and E-A176/G350*E7-prototype can be useful markers for predicting the progression of premalignant lesions and CC development.

4. Materials and Methods

4.1. Clinical Specimens and Diagnosis

The study enrolled 190 women, aged between 17 and 97 years old, who had all been identified as HPV 16 positive and were all residents of the Guerrero state in southern Mexico. Overall, 61 samples were from patients with CC, 6 samples were from patients with HSIL, 68 samples were from patients with LSIL, and 55 samples were from patients with no intraepithelial lesions. Cervical samples were obtained between 1997 and 2015 from cervical biopsies with histological diagnosis according to the FIGO classification system [65] or from cervical scrapes, with cytological diagnosis according to the Bethesda system [66]. Cervical scrapes (LSIL and no intraepithelial lesions) were collected using cytobrushes in a lysis buffer (10 mM Tris pH 8.0, 20 mM EDTA pH 8.0 and 0.5% sodium dodecyl sulfate) and the biopsies of women with HSIL or CC were immediately eluted in phosphate-buffered saline and stored at −70 °C until DNA extraction. DNA extraction was performed as previously described [67]. Briefly, the SDS-proteinase K-phenol-chloroform method [68] was used for DNA extraction from cervical scrapes and biopsies and the DNA was stored at −20 °C until analysis. The DNA integrity was evaluated by the amplification of the beta-globin gene [69]. All women without HPV 16 infection or from whom amplification of E6 and E7 genes was not obtained were excluded from the analysis. This study was approved by the Bioethical Committee of the Autonomous University of Guerrero and informed consent was obtained from all participants when the samples were collected.

4.2. Identification of HPV 16 E6/E7 Bicistronic Variants

E6 variants were identified as previously described [21]. HPV 16 E7 gene was amplified using the primers E7-F509 (5'-TGTATGTCTTGTTGACAGA-3') and E7-R917 (5'-CATCCATTACATCCCGTA-3'), which amplify a 409 bp region. Polymerase chain reaction (PCR) was performed in a final volume of 25 µL containing the DNA template, 2.5 µL of 10× PCR buffer, 0.3 mM dNTPs, 1 mM MgCl2, 0.36 µM of each primer, and 0.7 U Maxima Hot Start Taq DNA Polymerase (EP0602; Thermo Fisher Scientific, Waltham, MA, USA). For positive and negative controls, reactions with 300 ng of DNA from SiHa or CaSkI cells and H2O, respectively, were performed. PCR was performed using a Mastercycler EP Gradient (Eppendorf, Hamburg, Germany) as follows: initial denaturation at 95 °C for
4 min, followed by 40 cycles of 60 s at 95 °C for denaturation, 30 s at 57 °C for E7 for annealing, and 30 s at 72 °C for extension, with a final extension at 72 °C for 5 min. Correct amplification products were verified using DNA electrophoresis in an agarose gel.

Sequencing was performed using Big Dye Terminator V3.1 Cycle Sequencing (Applied Biosystems, Waltham, MA, USA) and the PCR products were purified using 75% isopropanol and loaded onto an ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer’s instructions. All sequences were analyzed and aligned using Finch TV v. 1.4.0 (Geospiza Inc., Denver, CO, USA) and William Pearson’s LALIGN (https://embnet.vital-it.ch/software/LALIGN_form.html, accessed on 19 June 2021), respectively. The FASTA sequence of the reference HPV 16 NC 1526.3 was used for the alignment and for the identification of E7 variants. Sequence data are publicly available in the GenBank database (accession numbers: MW452666-MW452855).

4.3. Statistical Analysis

Data was analyzed using the STATA v.11 software (StataCorp, College Station, TX, USA). Chi-square test was performed for analyzing E7 and E6/E7 genetic variants and lesions. The age-adjusted ORs and 95% CI were calculated for the E7 variant and E6/E7 bicistronic variants. Statistical significance was set at $p < 0.05$. 

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Institutional Review Board Statement: This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Bioethical Committee of the Universidad Autónoma de Guerrero.

Informed Consent Statement: Informed consent was obtained from all participants.

Data Availability Statement: Data generated and analyzed in this study are available upon request from the authors. The sequence data are available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 19 June 2021) with the accession numbers MW452666-MW452855.

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