Prevalence of HPV-16 genomic variant carrying a 63-bp duplicated sequence within the E1 gene in Slovenian women

Ž. Bogovac, M. M. Lunar, B. J. Kocjan, K. Seme, N. Jančar, M. Poljak

KEY WORDS

HPV-16, cervical cancer, E6-T350G variant

ABSTRACT

High-risk HPV, particularly HPV-16, is etiologically associated with the development of cervical cancer and its precursor lesions - cervical intraepithelial neoplasia (CIN). However, most precancerous lesions will not progress to cancer. Numerous studies have shown that HPV-16 consists of several genomic variants, which differ in their association with cervical cancer, viral persistence and the frequency of recurrence of cervical disease. Recently, a novel, presumably less pathogenic, HPV-16 E6-T350G genomic variant has been identified, carrying a 63-bp in-frame insertion in the E1 gene. No data from Slovenian patients have so far been reported for this specific HPV-16 variant. In the present study, therefore, a total of 390 HPV-16 positive samples obtained from the same number of women with normal cytology, CIN I, CIN II, CIN III or cervical cancer, were analyzed. The HPV-16 E1 insert variant was detected using real-time PCR-amplification of a 146-210-bp fragment of the E1 gene and PCR-sequencing of a 169-bp fragment of the E6 gene. The HPV-16 E1 insert variant was identified in 7/48 (14.6%), 1/21 (4.8%), 2/20 (10.0%), 9/131 (6.9%) and 12/170 (7.1%) of women with normal cytology, CIN I, CIN II, CIN III and cervical cancer, respectively. All HPV-16 E1 insert variants with an amplifiable E6 gene belonged to the European HPV-16 E6-350G variant group. No statistically significant differences in the prevalence of HPV-16 E1 insert genomic variant in women presenting with normal cytology and those with the different stages of HPV-16-induced disease were found.

Introduction

Human papillomavirus genotype 16 (HPV-16) is the major etiological agent of cervical cancer, accounting for 50-60% of cases globally, and also the HPV genotype most commonly found in women with its precursor lesions - cervical intraepithelial neoplasia (CIN), as well as in women with normal cytology (1, 2).

The genomic diversity of HPV-16 has been particularly extensively studied and shown to be substantial. Molecular-epidemiological studies have shown that HPV-16 consists of several genomic variants, which phylogenetically segregate and form five major genet-

ic lineages: European, Asian-American, two African branches (Af1 and Af2) and an Asian branch (3). Multiple studies have demonstrated that HPV-16 genomic variants differ in their association with cervical cancer, viral persistence and the frequency of recurrence of cervical disease. For instance, it has been shown in populations of North and Latin America and Asia that non-European HPV-16 variants are associated with stronger oncogenic potential than the European HPV-16 variants (3, 4).

A novel HPV-16 genomic variant was recently identified in Croatia, which harbored a 63-bp in-frame insertion (duplication) in the E1 gene; specifically, this variant was identified in 11.2% (48/429) of analyzed HPV-16 isolates (5). A comparison of cervical lesion severity of Croatian women with the wild-type or HPV-16 E1 insert gene showed that women with high-grade disease had a significantly lower prevalence of insert variant, suggesting that the HPV-16 strains harboring this particular duplication might have reduced oncogenicity. In addition, sequence analysis revealed that all HPV-16 E1 insert variants for which the E6/E7 genomic region was analyzed, had sequences identical to the European HPV-16 variant, with T at the nt position 350 replaced with G, often referred to as HPV-16 E6-T350G or the L83V variant (5). Several studies have addressed the possibility that HPV-16 E6-T350G may differ in its pathogenicity from the HPV-16 prototype, because of its increased association with persistent HPV infections (3).

In order to investigate the prevalence of the HPV-16 E1 insert genomic variant in Slovenian women, a total of 390 HPV-16 positive samples from women presenting with different stages of HPV-induced disease were analyzed in this study. Identification and characterization of the HPV-16 E1 insert variant was based on real-time (RT-) PCR-amplification of part of the E1 gene and PCR-sequencing of part of the E6 gene.

Materials and methods

A total of 390 HPV-16 positive clinical samples obtained from the same number of patients were in-

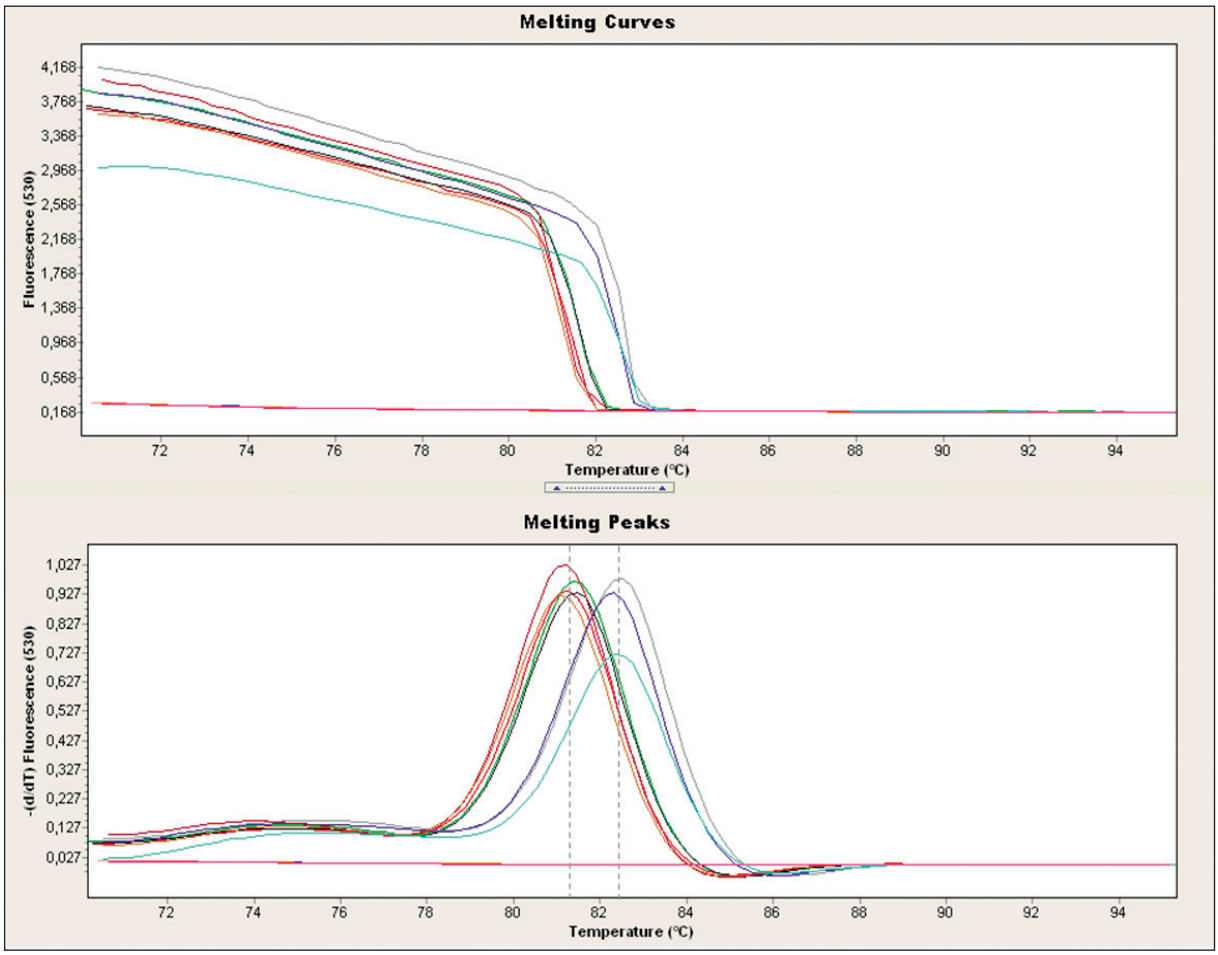

Figure 1. Melting curve analysis of the RT-PCR amplicons of HPV-16 E1 wild-type (146-bp; Tm=81.5°C) and mutant strains (210-bp; Tm=82.5°C).

cluded in the study; all samples originated from our previous studies on HPV in Slovenia and contained HPV-16 as the single infection (6-8). According to the cytological/histological results, 48 women had normal cytology, 21 women had CIN I, 20 women had CIN II, 131 women had CIN III and 170 women had invasive cervical cancer.

In order to discriminate HPV-16 E1 variants, a primer pair 1258f (5'-GCG GGT ATG GCA ATA CTG AA-3', nt-1258–1277) and 1404r (5'-TAA CAC CCT CTC CCC CAC TT-3', 1385–1404) was designed based on the HPV-16 prototype genome (K02718), to amplify a 146-bp (HPV-16 E1 wild-type variant) or 210-bp (HPV-16 E1 insert variant) fragment of the HPV-16 E1 gene. Identification of HPV-16 E1 variants was done by RT-PCR assay, which was performed in a 20 µl reaction mixture containing water, 100 ng of template DNA, 10 µl QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) and 0.5 µM of 1258f and 1404r primers. The assay was performed on a LightCycler 1.5 RT-PCR Instrument (Roche Diagnostics GmbH, Mannheim, Germany), under the following conditions: 95°C for 15 min (temperature transition rate of 20°C/s), followed by 40 cycles of 94°C for 15 s (20°C/s) and 60°C for 1 min (2°C/s). RT-PCR amplification was followed by melting curve analysis, which consisted of four temperature steps: 0 s at 95°C (20°C/s) and 30 s at 70°C (20°C/s), and then 0 s at 95°C (0.1°C/s), with continuous monitoring of the fluorescence. The final step consisted of cooling to 40°C (20°C/s) with a 30 s hold.

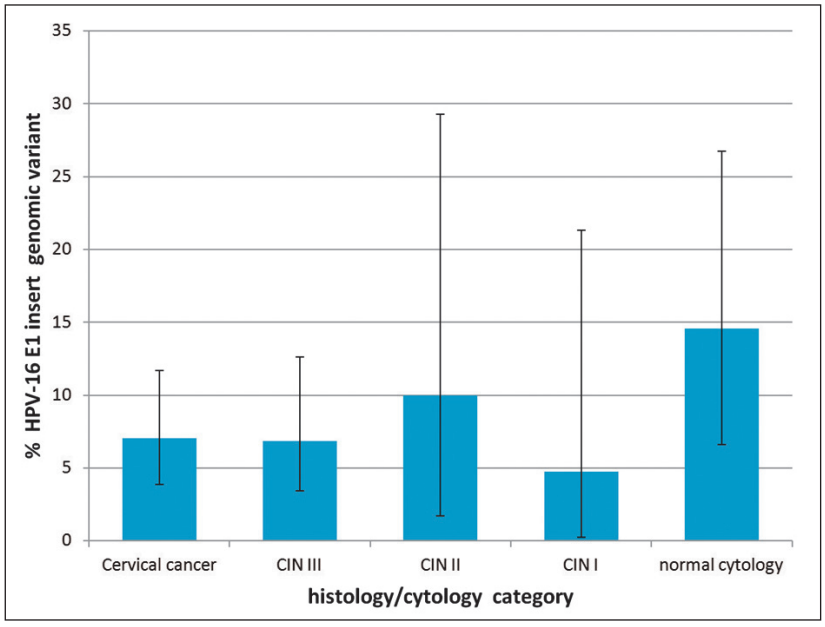

Figure 2. Prevalence of the HPV-16 E1 insert genomic variant with corresponding 95% confidence intervals in women with normal cytology and those with the different stages of HPV-16-induced disease.

Expected melting peaks for HPV-16 E1 wild-type variants and HPV-16 E1 insert variants were 81.5°C and 82.5°C, respectively.

In order to determine E6-T350G polymorphism among the HPV-16 E1 insert variants, a primer pair g16f-273 (5'-GGA ATC CAT ATG CTG TAT GTG-3', 273–293) and g16r-441 (5'-TCA GGA CAC AGT GGC TTT T-3', 423–441) was designed to amplify a 169-bp region of the HPV-16 E6 gene, including the polymorphic site. The PCR protocol was set up on a GeneAmp® PCR System 9700 (PE Applied Biosystems, Foster City, USA) and performed using a HotStarTaq *Plus* DNA Polymerase kit (Qiagen). The reaction mixture contained 100 ng of template DNA, 2.5 µl of 10× CoralLoad PCR Buffer with 15 mM $MgCl_2$, 200 µM of dNTPs, 0.625 U of HotStarTaq *Plus* DNA polymerase, 0.2 µM of each primers and water up to 25 µl. The cycling conditions used were 95°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1 min. This was followed by a final extension step at 72°C for 10 min and cooling of the reaction mixture to 4°C. PCR products that appeared as visible bands of the expected size were purified by a QIAquick PCR purification kit (Qiagen) and further processed for sequence analysis, as described previously (6).

Statistical analysis was done using Fischer's exact test and the Chi-square test (MedCalc 12.0.4.0 for Windows, MedCalc Software bvba, Mariakerke, Belgium). P-values were two-sided and considered statistically significant at a level of $P<0.05$, 95% confidence limits for proportions were calculated using on-line statistical package Epi Info™ Version 3.5.3 (http://www.openepi.com/OE2.3/Proportion/Proportion.htm).

Results

Using HPV-16 E1 type specific RT-PCR, HPV-16 was successfully amplified from all 390 samples included in the study. Melting curve analysis showed the presence of the HPV-16 E1 insert genomic variant in a total of 31/390 (8.0%) samples (Figure 1). Specifically, the prevalence of the 16 E1 insert genomic variant was 14.6% (7/48; 95% confidence interval (CI) for proportions, 6.61-26.73%) in women with normal cytology, 4.8% (1/21; 95% CI, 0.42-21.32%) in women with CIN I, 10.0% (2/20; 95% CI, 1.71-29.29%) in women with CIN II, 6.9% (9/131; 95% CI, 3.40-12.64%) in women with CIN III and 7.1% (12/170; 95% CI, 3.88-11.69%) in women with cervical cancer (Figure 2). Although some trend of decreasing prevalence of the HPV-16 E1 insert genomic variant with the severity of lesions was observed, no statistically si-

gnificant differences in the prevalence of the HPV-16 E1 insert genomic variant in women presenting with normal cytology and those with the different stages of HPV-16-induced disease, including cervical cancer, were found (Figure 2).

In order to confirm the specificity of the newly developed genotyping assay, HPV-16 E1 RT-PCR amplicons of 25 wild-type and 15 insert variants were sequenced. Complete agreement was observed between the results of the novel assay and PCR-sequencing. As previously described (5), the 63-bp insertion (duplicate) was found after genomic position 1311 according to the HPV-16 prototype (K02718).

Amplification and sequencing of the 169-bp fragment of E6 gene was successful in 30/31 (96.8%) HPV-16 E1 insert genomic variants, which all corresponded to the European HPV-16 E6-T350G variant lineage.

Discussion

The results of our study confirmed the previous observation of Sabol et al. (5) that infection with the HPV-16 E1 insert genomic variant is relatively rare in this geographical region. Altogether, only 8% of 390 HPV-16 positive clinical samples contained HPV-16 isolates with a 63-bp insertion (duplication) in the E1 gene. When women with normal cytology were compared with women with CIN III lesions or/and cervical cancer, the latter two groups had almost half the prevalence of the HPV-16 E1 insert variant, although these differences did not reach statistical significance in our study. This is probably due to wide confidence intervals as a consequence of the relatively small number of patients in each study group and relatively low prevalence observed. A similar falling trend in the prevalence of the HPV-16 E1 insert variant with the severity of CIN was also observed by Sabol et al. (5), who found the HPV-16 E1 insert variant in 13% (8/62), 12% (9/76) and 6% (5/80) of women with CIN I, CIN II and CIN III, respectively. However, Sabol et al. (5) did not provide confidence intervals in their paper, making a proper scientific judgment of the observed difference in prevalence of the HPV-16 E1 insert variant between study groups impossible. In addition, their entire CIN diagnosis was based on cytology and not histology (gold standard for CIN classification), opening a wide possibility of misclassification of study subjects. Finally, in contrast to our study, the Sabol et al. study (5) lacked a group of women with normal cytology and a group of women with cervical cancer, which are indispensable in any research of HPV pathogenesis. In order to resolve finally the question of whether the HPV-16 E1 insert variant has decreased oncogenicity compared to the HPV-16 E1 wild-type strains, future studies are required with many more enrolled subjects, especially women with normal cytology.

Sequence analysis revealed that all 30 successfully analyzed HPV-16 E1 insert variants had partial E6 sequence identity to the European HPV-16 genomic variant with the nt substitution T350G (L83V) in the E6 gene. Although this variant has been associated with viral persistence and disease progression in some countries, conflicting results have been found in others (reviewed in 3). In Slovenia, the HPV-16 E6-T350G variant accounts for about 60% of cases of cervical cancer (4). However, the observed differences between the pathogenicity of viral genomic variants characterized as HPV-16 E6-T350G in different European populations might be assigned to the new sublineage containing the 63-bp duplication within the E1 gene (5). Further studies are therefore needed to define the exact prevalence of this duplicate variant among HPV-16 E6-T350G isolates.

The underlying mechanism for the proposed reduced pathogenicity of the HPV-16 E1 insert variant is currently unknown. The HPV E1 protein is a helicase, ATPase and ATP-binding protein (9-11). Associated with the E2 protein, it attaches to *ori* in the LCR region of the HPV genome, forming an E1/E2 complex, which facilitates initiation of viral genome amplification (11-13). Any kind of genetic disturbance, therefore, such as insertions or deletions in the E1 gene, could theoretically affect the biological function of the E1 protein and, consequently, viral replication and pathogenesis.

In conclusion, the results of our study and the previous study by Sabol et al. (5) suggest that the HPV-16 genomic variant with a 63-bp insertion in the E1 gene might have reduced pathogenicity compared to the HPV-16 E1 wild-type strains, but further research is needed finally to clarify this issue.

REFERENCES

1. de Sanjose S, Diaz M, Castellsague X, Clifford G, Bruni L, Muñoz N, Bosch FX. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: A meta-analysis. Lancet Infect Dis 2007;7:453-9.

2. Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, Clifford GM Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: A meta-analysis update. Int J Cancer 2007; 121:621-2.

3. Bernard HU, Calleja-Macias IE, Dunn ST. Genome variation of human papillomavirus types: phylogenetic and medical implications. Int J Cancer 2006;118:1071-6.

4. Vrtačnik Bokal E, Kocjan BJ, Poljak M, Bogovac Ž, Jančar N. Genomic variants of human papillomavirus genotypes 16, 18, and 33 in women with cervical cancer in Slovenia. J Obstet Gynaecol Res 2010;36:1204-13.

5. Sabol I, Matovina M, Gasperov NM, Grce M. Identification of a novel human papillomavirus type 16 E1 gene variant with potentially reduced oncogenicity. J Med Virol 2008;80:2134-40.

6. Jančar N, Kocjan BJ, Poljak M, Lunar MM, Bokal EV. Distribution of human papillomavirus genotypes in women with cervical cancer in Slovenia. Eur J Obstet Gynecol Reprod Bio 2009;145:184-8.

7. Kovanda A, Juvan U, Šterbenc A, Kocjan BJ, Seme K, Jančar N, Vrtacnik-Bokal E, Poljak M. Pre-vaccination distribution of HPV genotypes in women with cervical intraepithelial CIN 3 lesions in Slovenia. Acta Dermatovenerol Alp Panonica Adriat 2009;18:47-52.

8. Poljak M, Oštrbenk A, Seme K, Učakar V, Hillemanns P, Bokal EV, Jančar N, Klavs I. Comparison of clinical and analytical performance of the Abbott Realtime High Risk HPV test to the performance of hybrid capture 2 in population-based cervical cancer screening. J Clin Microbiol 2011;-49:-1721-9.

9. Seo YS, Muller F, Lusky M, Hurwitz J. Bovine papilloma virus (BPV)-encoded E1 protein contains multiple activities required for BPV DNA replication. Proc Natl Acad Sci U S A 1993;90:702-6.

10. Park JS, Chee YH, Namkoong SE, Han SK, Kim TE, Lee HY, Kim SJ. Human papillomavirus detection in cervical carcinoma tissues and para-aortic lymph nodes by the polymerase chain reaction. Gynecol Oncol 1994;53:344-51.

11. Stanley MA, Pett MR, Coleman N. HPV: from infection to cancer. Biochem Soc Trans 2007;35:1456-60.

12. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond) 2006; 110:525-41.

13. Amin AA, Titolo S, Pelletier A, Fink D, Cordingley MG, Archambault J. Identification of domains of the HPV11 E1 protein required for DNA replication in vitro. Virology 2000;272:137-50.

AUTHORS' ADDRESSES

Željka Bogovac, BSc, National Forensic Laboratory, Ministry of the Interior, Štefanova ulica 2, 1501 Ljubljana, Slovenia

Maja M. Lunar, BSc, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1105 Ljubljana, Slovenia

Boštjan J. Kocjan, PhD, same address

Assoc. Prof. Katja Seme, MD, PhD, same address

Nina Jančar MD, PhD, Department of Obstetrics and Gynecology, University Medical Centre Ljubljana, Šlajmerjeva 3, 1000 Ljubljana, Slovenia

Prof. Mario Poljak, MD, PhD, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1105 Ljubljana, Slovenia. E-mail: mario.poljak@mf.uni-lj.si. Phone: +386 1 543 74 53