Journal of Medical Virology

Identification of a novel Human papillomavirus type 16 E1 gene variant with potentially reduced oncogenicity

Journal:	*Journal of Medical Virology*
Manuscript ID:	JMV-08-0818.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	15-Jul-2008
Complete List of Authors:	Sabol, Ivan; Rudjer Boskovic Institute, Molecular Medicine Matovina, Mihaela; Rudjer Boskovic Institute, Molecular Medicine Milutin Ga□perov, Nina; Rudjer Boskovic Institute, Molecular Medicine Grce, Magdalena; Rudjer Boskovic Institute, Molecular Medicine
Keywords:	Human papillomavirus 16, E1 variant , cervical cancer development, E6-350G variant

Identification of a novel Human papillomavirus type 16 E1 gene variant with potentially reduced oncogenicity

Running title: HPV 16 E1 gene variant

Ivan Sabol', Mihaela Matovina', Nina Milutin Gasperov, Magdalena Grce*

Rudjer Boskovic Institute, Division of Molecular Medicine, Zagreb, Croatia

This is the address of the institute and the affiliation of all authors

'Equally contributing authors

* Corresponding author:

Bijenicka cesta 54, 10000 Zagreb, Croatia

Tel: +385 1 456 1110

Fax: +385 1 456 1010

E-mail: grce@irb.hr

Abstract

The *Human papillomavirus* (HPV) 16 genome has been extensively studied, although no study has focused on the E1 gene that is implicated in viral DNA replication. After analyzing the E1 region of HPV 16 genomes in 429 cervical samples, 11.2% were found to contain a 63 nucleotides duplication in this region. Sequence analysis of the E6 and the E7 regions has shown that all samples containing this duplication were related to E6-G350 variant of the HPV 16 (Chi square test, p=0.0012). A comparison of cervical lesion severity of the examinees having regular or variant E1 genes has shown that the variant group had a significantly (Fischers exact test, p=0.0401) lower percentage of high grade disease cases, suggesting that this particular duplication might reduce the oncogenic potential of HPV 16, and also might clarify the differences of E6-G350 variant oncogenicity observed in European populations. Albeit, a decreased incidence of high grade cervical lesions can be linked to the prevalence of multiple HPV infection, the additional decrease of those cases with the variant E1 gene *versus* those without (10.5% and 18.6%, respectively) can only be ascribed to the effect of this particular HPV variant. Further research is needed to clarify the biology of these HPV 16 E1 variants.

Key words

Human papillomavirus 16, cervical cancer development, E6-350G variant, E1 variant

Introduction

Within the family *Papillomaviridae*, double stranded DNA viruses, more than 100 *Human papillomavirus* (HPV) types are well-characterized based on their genome sequences [de Villiers et al., 2004; Campo 2006]. Of about 40 mucosal (genital) HPV types, approximately 15 are classified as high risk types; they are the necessary etiological factor of cervical cancer, which is the second most common female cancer after breast cancer [Muñoz et al., 2003]. Of all high risk HPV types, HPV 16, genus *Alphapapillomavirus*, is the most commonly found in nearly 60% of cervical cancer cases worldwide [Clifford et al., 2003b]. It is also known that only a small percentage of HPV 16 positive lesions progress to cancer [Moreno et al., 1995; Bosch et al., 2002]; the reason for this still needs to be elucidated.

HPV variants are defined as viruses that vary by 2% or less in specified regions of the genome [Bernard et al., 1994], and some have been shown to display different oncogenicity (reviewed in Bernard et al., 2006). Most of the studies on HPV 16 variability were concerned with E6 and E7 proteins that are known HPV oncogenes. In addition, while many studies referred to the variability of E2, L1, L2 genes and LCR region, none were found on E1 variability or its relationship with HPV 16 oncogenicity (reviewed in Giannoudis and Herrington, 2001). The E1 gene encodes a multifunctional protein that can bind DNA and interact with the E2 protein and DNA polymerase alpha-primase [Chow and Broker, 2006; Wilson et al., 2002]. Furthermore, the E1 protein has ATPase and helicase activities [Hughes and Romanos, 1993] and together with the HPV E2 protein, plays an important role in HPV replication and transcriptional regulation [Chow and Broker, 1994]. Although the E1 gene seems to be relatively well conserved in

most HPV types [Shih-Yen et al., 1992], the goal of this study was to investigate a novel HPV type 16 variant within the E1 gene.

Materials and methods

Clinical samples

DNA samples were chosen from a collection of cervical specimens gathered previously from women attending the outpatient units of several gynaecology clinics in Zagreb (Croatia) for the purpose of HPV detection and genotyping in accordance with the Croatian protocol for the management of women with precancerous cervical lesions [Ljubojevic et al., 2001]. The cytological diagnosis of cervical specimens was determined according to the Croatian classification of cervical smears "Zagreb 2002" [Ovanin-Rakic et al., 2003] that is in line with the "2001 Bethesda" system for reporting Pap test results. In accordance with examinee cytological diagnosis cervical samples were grouped as follows: 25.4% (109/429) with atypical squamous cells of unknown significance (ASCUS), 20.5% (88/429) with low-grade squamous intraepithelial lesions (LSIL, also designed as cervical intraepithelial neoplasia grade 1, i.e. CIN1), 24.5% (105/429) with high-grade SIL/CIN2, 23.8% (102/429) with HSIL/CIN3 diagnosis, and 5.8% (25/429) of the samples with an unknown diagnosis. For 404 examinees, the age ranged from 17 to 69 with an average age of 25, while in 25 cases the age was unknown.

HPV detection and typing were performed with consensus and type-specific polymerase chain reaction (PCR) reactions as described previously [Grce et al., 2001]. Briefly, each cervical DNA specimen is amplified by three sets of consensus primers, and type-specific primers for the two most common low-risk HPV types 6 and 11 (common primer pair 6/11) that can be expected in the genital specimens, and also the four most common high-risk HPV types 16, 18, 31 and 33 that can be expected in high-grade cervical lesions [Clifford et al., 2003a].

In this study, a subset of 429 HPV 16 positive samples was analysed further. A total of 307 out of 429 (71.6%) samples were positive for probably only HPV 16 (other less common types, that were not detectable by the assay used, cannot be excluded), while 122 (28.4%) samples were positive for HPV 16 and at least one other HPV type determined by type-specific PCR (i.e. multiple HPV infection: HPV 16 + types 6/11, 18, 31 and/or 33; additional other less common HPV types cannot be excluded). The study was approved by the Ethical Board of the Rudjer Boskovic Institute.

DNA amplification of the E1 gene of HPV 16 DNA

In a previous investigation of HPV 16 viral genome integration (data submitted for publication), the amplification of E1/E2 region by primers designed by Lukaszuk et al. [2003] showed unexpectedly a different amplicon size from that anticipated in approximately 10% of samples (data was not presented). To analyze these unusual PCR products this study was performed.

A pair of 16E1-F (5'-CAT AGA GAT GCA GTA CAG G) and 16E1-R (5'-CTC ACC CCG TAT AAC TC) primers was designed in order to amplify the 417 bp from nucleotides 1099 to 1516 of the HPV 16 E1 gene. The PCR was performed in 20 µl containing 100 ng of sample DNA, PCR buffer (Promega), 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.25 µM of each primer and 0.125 U GoTaq DNA polymerase (Promega). Each PCR was carried out (Thermal cycler 2720, Applied Biosystems) under the following conditions: initial denaturation for 10 minutes at 95°C, 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 55°C and elongation for 45 seconds (+1 second for each cycle) at 72°C, and final elongation at 72°C for 7 minutes.

Aliquots (10 µl) of each PCR product were resolved by electrophoresis in a 2% agarose gel stained with ethidium bromide. The amplicons were identified by UV irradiation of the gels, and photographed by Image Master VDS (Pharmacia Biotech).

DNA Amplification of the E6/E7 region

For the analysis of the E6 region, the following primer pair was used: 16E6-F (5'-CAC AGT TAT GCA CAG AGC TGC) and 16E6-R (5'-CAT ATA TTC ATG CAA TGT AGG TGT A), amplifying 457 bp region from 141 to 597 of the E6 gene [Sotlar et. al, 2004]. The 20 µl reaction mixture contained 1X PCR buffer (Promega), 2.0 mM $MgCl_2$, 200 µM of each dNTP, 0.15 µM of each primer and 0.125 U GoTaq DNA polymerase (Promega). After the first 5 minutes of denaturation at 95^0C, 40 cycles of denaturation at 94^0C for 30 seconds, annealing at 57.5^0C for 30 seconds, and extension at 72^0C for 30 seconds were applied, followed by final elongation for 7 minutes at $72\ ^0$C.

Additionally, a second primer pair, 16E6/7-F (5'-CCC ACT GCT GGA CAA CAT GC) and 16E6/7-R (5'-GTG TGC CCA TTA ACA GGT CTT CCA), was used in order to amplify a product of 253 bp at the junction of the E6 and E7 regions, from 548 to 801. The 20 µl reaction mixture contained 1X PCR buffer (Promega), 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.15 µM of each primer and 0.125 U GoTaq DNA polymerase (Promega). After the first 5 minutes of denaturation at 95^0C, 35 cycles of denaturation at 94^0C for 15 seconds and annealing-extension at 62^0C for 4 minutes were applied, followed by final elongation for 7 minutes at $72\ ^0$C.

In these cases too, aliquots (10 µl) of each PCR product were resolved by electrophoresis in a 2% agarose gel stained with ethidium bromide and analysed on Image Master VDS (Pharmacia Biotech).

DNA sequencing and sequence analysis

Approximately 500 ng of each purified PCR product was sequenced at the local core sequencing facility (ABI PRISM 310 Genetic Analyzer, Applied Biosystems) of the Rudjer Boskovic Institute (Zagreb, Croatia), using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with both forward and reverse primers. The 7.0.5.2 Bioedit version was used for sequence alignment and translation. The referent HPV 16 genome sequence was obtained from the NCBI GenBank under accession number K02718, along with sequences of major HPV 16 variants [Yamada et al., 1997]: Asian-American (gb|AF402678), East Asian (gb|AF534061), African-1 (gb|AF536180), African-2 (gb|AF472509), and European German (gb|AF536179.1). The referent HPV16 E1 protein sequence was obtained from the NCBI protein database under accession number NP_041327.1.

Statistical analysis

The findings were processed manually into Microsoft Excel on a personal computer and the statistical analysis was performed using Fischer's exact test and Chi-square test (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA). The p values <0.05 were considered significant.

Results

Upon electrophoresis of the E1 HPV 16 amplicons, 88.6% (380/429) of samples had a regular band of expected amplicon size (417 bp), while 11.2% (48/429) had a variant band of approximately 480 bp in size (Table 1). Additionally, there was one sample (0.2%) containing both regular and variant band.

Eight samples showing larger and 2 samples showing regular E1 HPV 16 amplicons were sequenced to validate the difference in amplicon size and to determine sequence alignment. The sequencing showed that a duplication of 63 nucleotides was present in samples with a larger amplicon, i.e. variant band. A "monomer" sequence of the regular amplicon begins at position 1311 and ends at position 1373, while all variant amplicons contain the "monomer" sequence duplicated in tandem (Figure 1-A).

The E1 gene containing the observed insertion of 63 nucleotides, when translated should give rise to an enlarged protein of 670 amino acid residues long. The duplication is "in frame" in all respects in the DNA so the protein has the part of its sequence repeated. The amino acid sequence "monomer" is 21 residues long from amino acid at position 150 to position 170; the duplicated copy begins immediately afterwards (Figure 1-B).

To verify whether this duplication in the E1 region is a previously unknown feature of some already known HPV variant, parts of the E6 and E7 regions were sequenced in 9 of such samples. The E6 region sequencing revealed that every case resembled the HPV 16 European German type, which has G instead of T at position 350 of the genome, while they had no deviations from the referent HPV 16 (K02718 of the

GenBank) sequence in the E7 region; the European German variant had the same sequence as the referent variant in this region, so this was expected.

To be able to better understand the differences of samples with the 63 nucleotides duplication in relation to those with the regular E1 sequence, 13 samples without duplication were also analysed in the E6 and E7 regions. For the E6 region, 8 samples were found to be the referent HPV 16 type, 4 were identified as European German variants, having the same substitution as the E1 variant samples, and one was not related to the European variants. Sequencing of the E7 region of the samples without duplication revealed 12 of them to be European variants, similar to the E1 variant samples, and the remaining one was again unrelated to European variants.

The association of E1 duplication with G350 mutation (9/9) appears statistically significant (p=0.0012, Chi-square test) when compared with the distribution of G350 mutation in samples without the E1 duplication (4/13).

The one case unrelated to European variants in both the E6 and E7 sequence analyses had several substitutions in the E6 region (T>A at 286; A>G at 289; C>T at 335; T>G at 350) suggesting it to be related, but not identical to Af1, Af2 or AA variants. It also had 2 additional substitutions in the E7 region, C instead of T at position 789 and G instead of T at position 795 of the HPV16 referent genome. However, even with these additional differences it was impossible to determine the exact variant group to which this sample belongs, because there were other sites in the sequence that were identical to the referent sequence and which should be different in either the Af1, Af2 or AA variants.

There was no significant (Chi-square, p=0.0647) association between multiple infections and variant status (Table 1). However, if the one sample containing both, regular and variant HPV 16 E1 gene is included in the group of both those with and those

without the 63 nucleotide duplication, the difference becomes significant with p=0.0445 (Chi-square). In addition, if this sample is only ascribed to the sample group with the variant HPV 16 E1 gene the difference is also significant and even slightly higher with p=0.0413 (Chi-square).

The distribution of regular or variant HPV 16 E1 gene according to examinee cytological diagnosis shows a significant (Fischers exact test, p=0.0401) association between the group of samples with the variant HPV 16 E1 gene and the decrease of the cervical lesion grades, especially from CIN1 to CIN3. On the other hand, an increase of the cervical lesion grade is observed in the group of samples with the regular HPV 16 E1 gene, but this is most apparent for samples containing a single HPV 16 infection and of lesser extent than the decrease observed for multiple infections with variant HPV 16 E 1 gene (Figure 2 and Table 2).

Discussion

As a relatively large proportion (11.2%) of the analysed samples was shown to have the 63 nucleotide duplication in the E1 gene of the HPV 16 genome, further investigation of this region and its biological properties are needed.

The region of the E1 gene in which the 63 nucleotide duplication was observed has no function associated with it, except inconclusively H1 histone binding [Wilson et al., 2002]. Swindle and Engler (1998) have shown that the first 185 N-terminal amino acids of HPV 11 E1 protein bind H1 histone. Therefore, the duplication of amino acids from 150 to 170 might influence this binding in affected HPV 16 E1 proteins. In addition, Amin et al. (2000) found that HPV E1 proteins lacking the N-terminal domain are less effective in supporting HPV replication than full length proteins. Additionally, Yasugi et al. (1997) earlier suggested that amino acids 144-420 also play a role in E1-E2 interactions at physiological temperatures, and this might be disrupted in samples harbouring the observed duplication.

To assess whether the variant E1 protein has any impact on the oncogenic potential of the supposed HPV 16 variant, samples were sorted in groups according to the presence of duplication. For both groups, data on cytological diagnosis and examinee age were retrieved from the database (Table 2). The group with the regular E1 gene was significantly less associated with CIN1 and more with CIN3 cases than the group with the variant E1 gene (Figure 2). As the mean age of both groups is the same, the age is unlikely to be the cause of this decrease in lesion grade.

Some authors link the presence of multiple HPV infections with an increased severity or increased risk of lesions of the cervices [van der Graaf et al., 2002; Ho et al., 1998; Morrisonet al., 1991; Sasagawaet al., 2001]. On the other hand, other studies report

no significant additional risk of cervical intraepithelial neoplasia cervical cancer when comparing patients with single or multiple infections [Bosch et al., 2002; Herrero et al., 2000]. In addition, Gargiulo et al. (2007) report finding multiple HPV infections significantly less often in severe dysplasia and cervical cancer. Thus, the presence of multiple infections in the sample pool of this study might confound these findings as a lower disease grade might be associated with multiple HPV infection and not variant HPV 16. To address this issue, samples were split depending on the status of HPV 16 infection (probable single and confirmed multiple with HPV 6/11, 18, 31 and/or 33). In the sample pool, the incidence of CIN3 was the highest in cases with probably single infection with HPV 16 without duplication, and the lowest in cases containing multiple infections including variant HPV 16 with duplication (Table 2 and Figure 2). The findings presented in this study are in line with the findings of Gargiulo et al. (2007), as when the group of samples with the variant HPV 16 E1 gene were disregarded, multiple infection samples were less associated with CIN3 cases than cases with probable single infections, but this difference was not significant (Fischer's exact test, p=0.2424). While a decreased incidence of CIN3 can be linked to the prevalence of multiple HPV infection, the additional decrease of CIN3 incidence in samples with multiple HPV infection and with the variant *versus* regular HPV 16 E1 gene (10.5% and 18.6%, respectively) can only be ascribed to the effect of this particular HPV 16 variant (Table 2).

Sequencing results revealed that all samples with the variant HPV 16 E1 gene for which E6/E7 was analysed, had sequences identical to the European German variant, having T at nucleotide 350 replaced with G (often referred to as E6-350G or L83V variant). As 4 of 13 samples with the regular HPV 16 E1 gene were also shown to be the European German variant with the same substitution, it is likely that the decreased

oncogenicity in the group of samples with the variant HPV 16 E1 gene comes from the reduced oncogenicity of the E6-350G subvariant containing the 63 nucleotides duplication in the E1 gene relative to "normal" E6-350G or referent HPV 16 variants. Moreover, as the group of samples with the regular HPV 16 E1 gene was mostly determined to be the European variant, the association of those samples with CIN3 is probably due the expected oncogenicity of the referent European HPV 16 variant [Zehbe et al., 1998a] and not due to the large presence of other more oncogenic variants.

This finding is interesting, as the "normal" E350G variant was previously described as either strongly associated with cervical cancer in some European populations like Swedish or French [Zehbe et al 1998a; Grodzki et al 2006] or not clearly associated with cervical cancer in others [Bontkes et al., 1998; Zehbe et al., 1998b; Brady et al., 1999; Nindl et al., 1999; van Duin et al., 2000]. It was suggested that genetic differences between populations, in particular in the human leukocyte antigens, contribute to this difference [Zehbe et al., 1998b, 2001]. Another study, focused on *in vitro* evaluation of E6 variants, has shown that this E350G variant has the same ability to degrade p53 and Bax levels as the referent E6 protein. However, this variant was shown to have a slightly increased capability of inhibiting serum/calcium induced differentiation, which led the authors to believe that this variant might have increased pathogenicity [Asadurian et al., 2007]. This *in vitro* study shows that the E350G variant does not have lesser oncogenicity due to functional changes to the E6 protein, eliminating this mutation as a source of disagreement between studies in different populations.

In this study, the E350G variant exhibits reduced oncogenicity, which is consistent with studies done in geographically closer populations. However, this effect was more clearly observed in samples with the variant HPV 16 E1 gene suggesting that

the different oncogenicity of variants characterized as E350G in different European populations might be assigned to the new subvariant containing the 63 nucleotide duplication within the E1 gene that was observed, or some other genome change in addition to possible genetic differences in European populations. It would be interesting to investigate whether the samples in studies showing no association between E350G and cervical cancer also contain this duplication in the E1 gene, as this region of the HPV genome was not evaluated at all.

The 63 nucleotide duplication within the E1 gene observed in this study was previously described only once by Dong et al. (2000), who analysed 28, HPV 16 positive, cervical cancer samples for other reasons in the Chinese population. From their unexpected chance observation and our focused study of this possible new variant, it seems to have a world-wide distribution and is not an isolated local case on either continent. Neither their group nor this one has followed the examinees after cervical specimen collection, so no firm conclusions regarding the long-term effect on the persistence of infection and its oncogenic potential are possible.

Data presented in this study suggests that the HPV 16 variant with the 63 nucleotide E1 duplication might exhibit decreased oncogenicity, especially in case of the HPV 16 G350 variant that was suggested to have increased oncogenicity. This study might also encourage further research in the up-to-now neglected variability of the HPV 16 E1 region and its effect on disease progression.

Acknowledgements

This work was supported by grant numbers 098-0982464-2510/2007 from the Ministry of Science, Education and Sport of the Republic of Croatia. The authors would like to thank Jasminka Golubic-Talic, her expert technical assistance. The authors would also like to thank Dr. Helena Cetkovic for her helpful advice and assistance on DNA sequencing.

References

Amin AA, Titolo S, Pelletier A, Fink D, Cordingley MG, Archambault J. 2000. Identification of domains of the HPV11 E1 protein required for DNA replication in vitro. J Virol 272:137-150.

Asadurian Y, Kurilin H, Lichtig H, Jackman A, Gonen P, Tommasino M, Zehbe I, Sherman L. 2007. Activities of Human Papillomavirus 16 E6 Natural Variants in Human Keratinocytes. J Med Virol 79:1751-1760.

Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM. 1987. Structural and Transcriptional Analysis of Human Papillomavirus Type 16 Sequences in Cervical Carcinoma Cell Lines. J Virol 61:962-971.

Bernard HU, Calleja-Macias IE, Dunn ST. 2006. Genome variation of human papillomavirus types: Phylogenetic and medical implications. Int J Cancer 118:1071-1076.

Bernard HU, Chan SY, Manos MM, Ong CK, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM. 1994. Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. J Infect Dis 170:1077-1085.

Bontkes HJ, van Duin M, deGruijl TD, Duggan-Keen MF, Walboomers JM, Stukart MJ, Verheijen RH, Helmerhorst TJ, Meijer CJ, Scheper RJ, Stevens FR, Dyer PA, Sinnott P, Stern PL. 1998. HPV 16 infection and progression of cervical intraepithelial neoplasia: Analysis of HLA polymorphism and HPV16 E6 sequence variants. Int J Cancer 78:166-171.

Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. 2002. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 55:244-265.

Brady CS, Duggan-Keen MF, Davidson JA, Varley JM, Stern PL. 1999. Human papillomavirus type 16 variants in cervical carcinoma: Relationship to host genetic factors and clinical parameters. J Gen Virol 80:3233-3240.

Campo MS, editor. 2006. Papillomavirus Research - From Natural History to Vaccines and Beyond. Caister Academic Press, UK, 424 p.

Chow LT, Broker TR. 1994. Papillomavirus DNA replication Intervirology 37:150-158.

Chow LT, Broker TR. 2006. Mechanisms and regulation of papillomavirus replication. In: Campo MS, editor. Papillomavirus research - From natural history to vaccines and beyond. Norfolk, Kaister Academic Press, UK, p. 53-71.

Clifford GM, Smith JS, Aguado T, Franceschi S. 2003a. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. Br J Cancer 89:101-105.

Clifford GM, Smith JS, Plummer M, Munoz N, Franceschi S. 2003b. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br J Cancer 88:63-73.

de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. 2004. Classification of papillomaviruses. Virology 324:17-27.

Dong X, Zhou W, Pfister H. 2000. [Presence of genetic rearrangements in E1/E2 regions of episomal HPV 16 isolates from cervical carcinomas]. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi 14:5-8. [In Chinese]

Gargiulo F, De Francesco MA, Schreiber C, Ciravolo G, Salinaro F, Valloncini B, Mancaa N. 2007. Prevalence and distribution of single and multiple HPV infections in cytologically abnormal cervical samples from Italian women. Virus Res 125:176-182.

Giannoudis A, Herrington CS. 2001. Human papillomavirus variants and squamous neoplasia of the cervix. J Pathol 193:295-302.

Grce M, Husnjak K, Bozikov J, Magdic L, Zlacki M, Lukac J, Fistonic I, Sikanic-Dugic N, Pavelic K. 2001. Evaluation of genital human papillomavirus infections by polymerase chain reaction among Croatian women. Anticancer Res 21:579-584.

Grodzki M, Besson G, Clavel C, Arslan A, Franceschi S, Birembaut P, Tommasino M, Zehbe I. 2006. Increased risk for cervical disease progression of French women infected with the human papillomavirus type 16 E6-350G variant. Cancer Epidemiol Biomarkers Prev 15:820-822.

Herrero R, Hildesheim A, Bratti C, Sherman ME, Hutchinson M, Morales J, Balmaceda I, Greenberg MD, Alfaro M, Burk RD, Wacholder S, Plummer M, Schiffman M. 2000. Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica. J Natl Cancer Inst 92:464-474.

Ho GY, Palan PR, Basu J, Romney SL, Kadish AS, Mikhail M, Wassertheil-Smoller S, Runowicz C, Burk RD. 1998. Viral characteristics of human papillomavirus infection and antioxidant levels as risk factors for cervical dysplasia. Int J Cancer 78:594-599.

Hughes FJ, Romanos MA. 1993. El protein of human papillomavirus is a DNA helicase/ATPase. Nucleic Acids Res 21:5817-5823.

Ljubojevic N, Babic S, Audy-Jurkovic S, Ovanin-Rakic A, Jukic S, Babic D, Grubisic G, Radakovic B, Ljubojevic-Grgec D. 2001. Improved national Croatian dignostic and

therapeutic guidelines for premalignant lesions of the uterine cervix with some cost-benefit aspects. Coll Antropol 25:467-74.

Lukaszuk K, Liss J, Wozniak I, Emerich J, Wojcikowski C. 2003. Human papillomavirus type 16 status in cervical carcinoma cell DNA assayed by multiplex PCR. J Clin Microbiol 41:608-612.

Moreno V, Munoz N, Bosch FX, de Sanjose S, Gonzalez LC, Tafur L, Gili M, Izarzugaza I, Navarro C, Vergara A, Viladiu P, Ascunce N, Shah KV. 1995. Risk factors for progression of cervical intraepithelial neoplasm grade III to invasive cervical cancer. Cancer Epidemiol Biomarkers Prev 4:459-467.

Morrison EA, Ho GY, Vermund SH, Goldberg GL, Kadish AS, Kelley KF, Burk RD. 1991. Human papillomavirus infection and other risk factors for cervical neoplasia: a case-control study. Int J Cancer 49:6-13.

Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJF, Meijer CJLM. 2003. Epidemiological classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348:518-527.

Nindl I, Rindfleisch K, Kotz B, Schneider A, Durst M. 1999. Uniform distribution of HPV 16 E6 and E7 variants in patients with normal histology, cervical intraepithelial neoplsia and cervical cancer. Int J Cancer 82:203-207.

Ovanin-Rakic A, Pajtler M, Stankovic T, Audy-Jurkovic S, Ljubojevic N, Grubisic G, Kuvacic I. 2003. [Classification of cervical cytological smears "Zagreb 2002". Modification of the classification "Zagreb 1990" and "NCI Bethesda 2001".] Gynaecol Perinatol 12:148-153. [In Croatian]

Sasagawa T, Basha W, Yamazaki H, Inoue M. 2001. High-risk and multiple human papillomavirus infections associated with cervical abnormalities in Japanese women. Cancer Epidemiol Biomarkers Prev 10:45-52.

Seedorf K, Krammer G, Durst M, Suhai S, Rowekamp WG. 1985. Human Papillomavirus Type 16 DNA Sequence. Virology 145:181-185.

Shih-Yen C, Bernard HU, Chi-Keong O, Shih-Ping C, Hofmann B, Delius H. 1992. Phylogenetic Analysis of 48 Papillomavirus Types and 28 Subtypes and Variants: a Showcase for the Molecular Evolution of DNA Viruses. J Virol 66:5714-5725.

Sotlar K, Diemer D, Dethleffs A, Hack Y, Stubner A, Vollmer N, Menton S, Menton M, Dietz K, Wallwiener D, Kandolf R, Bultmann B. 2004. Detection and Typing of Human Papillomavirus by E6 Nested Multiplex PCR. J Clin Microbiol 42:3176-3184.

Swindle CS, Engler JA. 1998. Association of the human papillomavirus type 11 E1 protein with histone H1. J Virol 72:1994-2001.

van der Graaf Y, Molijn A, Doornewaard H, Quint W, van Doorn LJ, van den Tweel J. 2002. Human papillomavirus and the long-term risk of cervical neoplasia. Am J Epidemiol 156:158-164.

van Duin M, Snijders PJ, Vossen MT, Klaassen E, Voorhorst F, Verheijen RH, Helmerhorst TJ, Meijer CJ, Walboomer JM. 2000. Analysis of human papillomavirus type 16 variants in relation to p53 codon 72 polymorphism genotypes in cervical carcinogenesis. J Gen Virol 81:317-325.

Wilson VG, West M, Woytek K, Rangasamy D. 2002. Papillomavirus E1 proteins: form, function, and features. Virus Genes 24:275-90.

Yamada T, Manos MM, Peto J, Greer CE, Munoz N, Bosch FX, Wheeler CM. 1997. Human papilloma type 16 sequence variation in cervical cancers: a worldwide perspective. J Virol 71:2463-2472.

Yasugi T, Benson JD, Sakai H, Vidal M, Howley PM. 1997. Mapping and characterization of the interaction domains of human papillomavirus type 16 E1 and E2 proteins. J Virol 71:891-899.

Zehbe I, Wilander E, Delius H, Tommasino M. 1998a. Human papillomavirus 16 E6 variants are more prevalent in invasive cervical carcinoma than the prototype. Cancer Res 58:829-833.

Zehbe I, Voglino G, Delius H, Wilander E, Tommasino M. 1998b. Risk of cervical cancer and geographical variations of human papillomavirus 16 E6 polymorphisms. Lancet 352:1441-1442.

Zehbe I, Tachezy R, Mytilineos J, Voglino G, Mikyskova I, Delius H, Marongiu A, Gissmann L, Wilander E, Tommasino M. 2001. Human papillomavirus 16 E6 polymorphisms in cervical lesions from different European populations and their correlation with human leukocyte antigen class II haplotypes. Int J Cancer 94:711-716.

Table 1. Distribution of cervical specimens containing the regular amplicon and those containing a 63 nucleotides duplication in the HPV 16 E1 gene

Table 2. Distribution of examinee cytological diagnosis and age in different sample groups

Figure 1. A) Nucleotide sequence alignment of samples containing regular (RB) or variant band (VB) of the HPV 16 E1 gene in comparison with the referent HPV 16 sequence published in GenBank under accession number K02718; HPV 16 is the referent sequence; VB1-VB7 are representative sequences of samples with the 63 nucleotides duplication in the HPV 16 E1 gene; RB1 and RB2 are sequences of 2 samples with regular sequence alignment; the duplicated sequence is underlined in the HPV 16 and VB1 sequences. B) Protein sequence alignment of sequences derived by translating nucleotide sequences of VB samples (containing the 63 nucleotide duplication) to the referent HPV 16 E1 protein sequence from the NCBI protein database accessible under accession number NP_041327.1; the duplicated sequence is underlined

Figure 2. Incidence of A) regular and B) variant band (63 nucleotide duplication) of the HPV 16 E1 gene according to cytological diagnosis and HPV 16 status (probable single and multiple infection with confirmed HPV 6/11, 18, 31 and/or 33)

Table 1. Distribution of cervical specimens containing the normal and variant HPV 16 E1gene

	Number of cervical samples (%)			
	regular*	**variant****	**regular and variant**	**Total**
Single HPV 16 infection	278 (90.6)	29 (9.4)	0 (0)	307 (71.6)
Multiple HPV infection***	102 (83.6)	19 (15.6)	1 (0.8)	122 (28.4)
All cases	380 (88.6)	48 (11.2)	1 (0.2)	429 (100)

*regular HPV 16 E1 amplicon size (417 bp); **larger HPV 16 E1 amplicon size (480 bp) containing a 63 nucleotides duplication; ***HPV 16 with 6/11, 18, 31 and/or 33

Table 2. Distribution of examinee cytological diagnosis and age in different sample groups

Sample group*	Number of samples** (%)	Examinee cytological diagnosis***[27]					Patient age (years)	
		Unknown N (%)	ASCUS N (%)	LSIL CIN1 N (%)	HSIL CIN2 N (%)	HSIL CIN3 N (%)	Average age (range)	Unknown N (%)
Samples with normal E1 HPV 16 gene	380 (88.8%)	21 (5.5%)	101 (26.6%)	73 (19.2%)	91 (23.9%)	94 (24.7%)	29 (18-69)	23 (6.0%)
Single HPV 16 infection	278 (64.8)	12 (4.3)	70 (25.2)	54 (19.4)	67 (24.1)	75 (27.0)	30 (18-54)	19 (6.8)
Multiple HPV infection	102 (24.0)	9 (8.8)	31 (30.4)	19 (18.6)	24 (23.5)	19 (18.6)	28 (18-69)	4 (3.9)
Samples with variant E1 HPV 16 gene	48 (11.2%)	4 (8.3%)	8 (16.7%)	15 (31.3%)	14 (29.2%)	7 (14.6%)	29 (17-57)	2 (4.2%)
Single HPV 16 infection	29 (6.8)	3 (10.3)	4 (13.8)	8 (27.6)	9 (31.0)	5 (17.2)	28 (17-50)	1 (3.4)
Multiple HPV infection	19 (4.4)	1 (5.3)	4 (21.1)	7 (36.8)	5 (26.3)	2 (10.5)	30 (19-57)	1 (5.3)
Total	428 (100)	25 (5.8)	109 (25.5)	88 (20.6)	105 (24.5)	101 (23.6)	29 (17-69)	25 (5.8)

*normal HPV - regular HPV 16 E1 amplicon size (417 bp), variant HPV - larger HPV 16 E1 amplicon size (480 bp) containing a 63 nucleotides duplication, multiple HPV infection - HPV 16 with 6/11, 18, 31 and/or 33; **one sample with a normal and a variant HPV 16 E1 amplicon corresponding to a 23-year old woman with CIN3 and multiple HPV infection was excluded; *** ASCUS - atypical squamous cells of unknown significance, LSIL - low-grade squamous intraepithelial lesions, CIN1 - cervical intraepithelial neoplasia grade 1, HSIL - high-grade squamous intraepithelial lesions, CIN2/3 – CIN grade 2 or 3

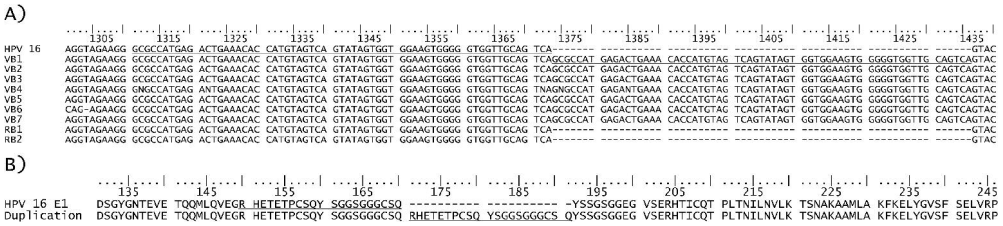
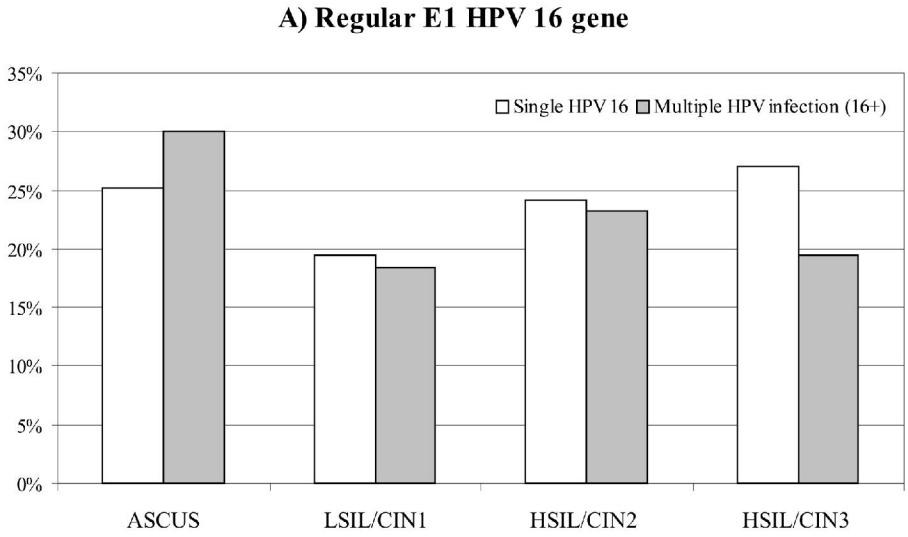


Figure 1. A) Nucleotide sequence alignment of samples containing regular (RB) or variant band (VB) of the HPV 16 E1 gene in comparison with the referent HPV 16 sequence published in GenBank under accession number K02718; HPV 16 is the referent sequence; VB1-VB7 are representative sequences of samples with the 63 nucleotides duplication in the HPV 16 E1 gene; RB1 and RB2 are sequences of 2 samples with regular sequence alignment; the duplicated sequence is underlined in the HPV 16 and VB1 sequences. B) Protein sequence alignment of sequences derived by translating nucleotide sequences of VB samples (containing the 63 nucleotide duplication) to the referent HPV 16 E1 protein sequence from the NCBI protein database accessible under accession number NP_041327.1; the duplicated sequence is underlined



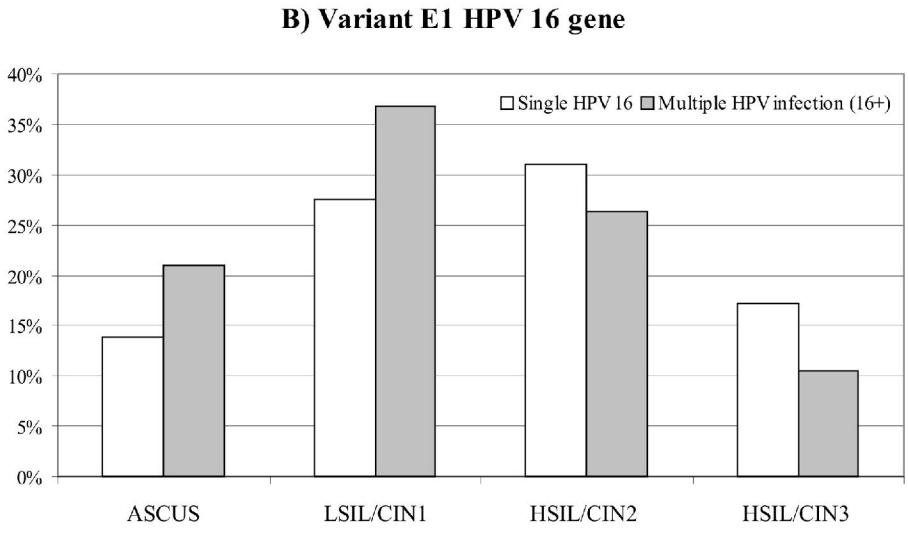


Figure 2. Incidence of A) regular and B) variant band (63 nucleotide duplication) of the HPV 16 E1 gene according to cytological diagnosis and HPV 16 status (probable single and multiple infection with confirmed HPV 6/11, 18, 31 and/or 33)