Sabol, I., Čretnik, M., Hadžisejdić, I., Si-Mohamed, A., Matovina, M., Grahovac, B., Levanat, S., Grce, M., 2009. A new approach for the evaluation of the human papillomavirus type 16 variability with high resolution melting analysis. Journal of Virological Methods 162, 142–147. doi:10.1016/j.jviromet.2009.07.029 – Accepted Version of Manuscript

A new approach for Human Papillomavirus Type 16 Variability Evaluation with High Resolution Melt Sequence Analysis

Ivan Sabol[a], Maja Čretnik[b], Ita Hadžisejdić[c], Ali Si-Mohamed[d], Mihaela Matovina[a], Blaženka Grahovac[c], Sonja Levanat[b], Magdalena Grce[a]*

a Laboratory of Molecular Virology and Bacteriology, Division of Molecular Medicine, Rudjer Boskovic Institute, Zagreb, Croatia

b Laboratory of Hereditary Cancer, Division of Molecular Medicine, Rudjer Boskovic Institute, Zagreb, Croatia

c Laboratory for Molecular Diagnostic, Department of Pathology, Rijeka University School of Medicine, Rijeka, Croatia

d Laboratoire de Virologie, Hôpital Européen Georges Pompidou, Paris, France

*Corresponding Author:

Magdalena Grce, Head, Laboratory of Molecular Virology and Bacteriology, Division of Molecular Medicine, Rudjer Boskovic Institute, Bijenicka cesta 54, 10002 Zagreb, Croatia.

Phone: +385-1-4561110. Fax: +385-1-4561010. E-mail: grce@irb.hr

Abstract

Studies on the variability of Human papillomavirus (HPV) type 16 are based mostly on DNA sequencing of the viral oncogenes E6 and E7. In order to simplify variant identification, high resolution melting (HRM) analysis, which has been shown to distinguish amplicons differing in a single nucleotide, was employed.

Optimised HRM analysis was applied to 255 anogenital samples positive for HPV 16. The E6/E7 region of the HPV 16 genome was amplified using nested PCR with subsequent melting of the amplicons. Samples giving ambiguous melting profiles were melted again in the presence of the reference HPV 16 DNA to define and confirm the novel melting profiles.

Out of 219 samples of Croatian origin, 65 reference variants, 119 E6-360G variants and 35 novel melting profiles were found. Samples containing unusual profiles were sequenced for identification. In addition, a subset of samples with two common variants, 23 reference and 34 E6-350G variants, was also sequenced to confirm the findings of high resolution melting. Concordance between melting analysis and sequencing was 93.9%, while HRM sensitivity and specificity were 92.9% and 94.7%, respectively.

This study showed that HRM analysis can be useful for the identification of HPV 16 variants. The HRM method will be useful in low resource settings as it saves considerable time and resources compared to sequencing.

Key words: human papillomavirus 16; variant; high resolution melting; sequencing

Abbreviations: HPV - human papillomavirus; PCR - polymerase chain reaction; HRM - high resolution melting; SNP - single nucleotide polymorphisms

1. Introduction

The Human papilloma viruses (HPV) constitute a group of small double stranded DNA viruses. More than 100 HPV types are known and about 40 infect the anogenital tract, of which 13 types, so-called high-risk types, cause cervical cancer and other malignancies (de Villiers et al., 2004; Munoz et al., 2003). Large epidemiological studies indicate a convincing association between cervical cancer and HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66; the evidence for carcinogenicity was the strongest for HPV 16, found in almost of 60% cervical cancer samples (Clifford et al., 2003; IARC, 2007). The most common HPV type discovered in other anogenital cancer samples was also HPV 16. In addition, each HPV type has many variants that differ from the sequence of the reference type by up to 2% and it was suggested that some variants have different carcinogenic potential (Bernard et al., 1994; Bernard et al., 2006).

Studies of the variability of HPV types are very laborious and costly as all samples in the study population need to be sequenced in selected regions of their genome and the resulting sequences need to be compared to the reference sequence to identify any mismatches. The high resolution melting (HRM) analysis consists of DNA amplification by the polymerase chain reaction (PCR) and subsequent melting of the amplicons in the presence of double-stranded DNA-binding dye and fluorescence detection. After data normalization, the sequence variation can be detected as changes in the shape of the resulting melting curves (Wittwer et al., 2003). The method enables the differentiation of single nucleotide polymorphisms (SNP) and, in optimal conditions, the specificity and the sensitivity of genotyping of the SNP reaches 100% for smaller amplicons (Reed and Wittwer, 2004).

The goal of this study was to implement the high resolution melting analysis for the identification of HPV 16 variants and to assess the distribution of those variants in Croatia.

2. Materials and methods

2.1. Anogenital samples

Archival DNA from cervical smears collected from women attending several outpatient gynaecology and obstetrics clinics in Zagreb (Croatia) were used in this study (Grce et al., 2001; Milutin-Gasperov et al., 2007). Cervical smears were previously collected from women with abnormal cytological findings for the purpose of detection and genotyping

of HPV (Ljubojevic et al., 2001). DNA was isolated from samples using standard phenol-chlorophorm method while HPV detection and genotyping were performed using consensus and type specific PCR primers (Grce et al., 2001). Herein, a subset of 212 archival samples positive for HPV 16 was further analysed.

Twenty seven fresh cervical cancer specimens that were positive for HPV 16 and collected at the University Hospital of Rijeka (Rijeka, Croatia) were also included in this study. Automated DNA extraction was performed using the NucliSENS easyMAG (bioMerieux, Marcy l'Etoile, France). Amplification and detection of HPV DNA was performed by using the Linear Array HPV genotyping test (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions. Briefly, after DNA isolation, HPV DNA was amplified using the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, California, USA) followed by fully automated hybridization and detection on the ProfiBlot 48 (Tecan Trading AG, Zurich, Switzerland).

In addition, sixteen more HPV 16 positive anogenital specimens from the European Hospital "Georges Pompidou" (Paris, France) were included in the study. Those samples, previously identified as HPV 16 by sequencing (Si-Mohamed et al., 2005), were used in this study as blind control to validate the method.

The study was approved by the Research Ethics Committee at the Rudjer Boskovic Institute of Zagreb.

2.2. DNA amplification

The E6 region of HPV 16 was amplified by nested PCR. Outer PCR primers were degenerate GP-E6-3F (5'-GGG WGK KAC TGA AAT CGG T; single-letter code: W= A/T; K= G/T; R= A/G; N= A/C/G/T) and GP-E6-5B (5' -CTG AGC TGT CAR NTA ATT GCT CA) amplifying HPV DNA from nucleotide 27 to 636 of the HPV 16 genome (Sotlar et al., 2004). The inner primers that gave the final amplicon were: 16ORF5' (5'-CGAAACCGGTTAGTATAA) and 16ORF3' (5'-GTATCTCCATGCATGATT) amplifying HPV 16 DNA from nucleotide 52 to 575 of the genome (Ortiz et al., 2006).

Initially 50 ng of DNA was amplified using the outer primer set in a 10 μL reaction containing 1X PCR buffer (Promega, Madison, Wisconsin, USA), 1500 μM $MgCl_2$, 200 μM each dNTP and 0.3 μM each GP-E6-3F and GP-E6-5B primers. The amplification protocol was as follows: initial denaturation of 4 minutes at 95°C followed by 40 cycles of

denaturation for 30 seconds at 95°C, annealing for 30 seconds at 56°C and elongation for 65 seconds at 72°C. The final cycle was followed by the final elongation step at 72°C for 7 minutes (Applied Biosystems Veriti thermal cycler, Foster City, California, USA).

Each amplicon of the first PCR was diluted 10X with PCR grade water and 1 µL was used in the nested PCR amplification using the inner primer set. The reaction consisted of 1X PCR buffer (Promega, Madison, Wisconsin, USA), 3000 µM $MgCl_2$, 100 µM each dNTP and 0.2 µM each 16ORF5' and 16ORF3' primers. In addition, 1 µl LC Green Plus dye (Idaho technologies, Salt Lake City, Utah, USA) was added to each reaction. The amplification protocol was: initial denaturation of 5 minutes at 95°C followed by 40 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 58°C and elongation for 30 seconds at 72°C. The final cycle was followed by the final elongation step at 72°C for 7 minutes (Applied Biosystems Veriti thermal cycler, Foster City, California, USA).

2.3. HRM analysis

The amplicons of the second PCR were transferred to LightCycler 20 µl capillaries (Roche Applied Science, Indianapolis, Indiana, USA) and melted in HR-1 instrument (Idaho technologies, Salt Lake City, Utah, USA) with the ramping speed of 0.2°C/s and fluorescence data collection from 77°C to 86°C. The resulting data was analyzed with the HR-1 melt analysis tool (Idaho technologies, Salt Lake City, Utah, USA). All melting curves were normalized and temperature shifted, and the first derivative plots were compared.

2.4. Heteroduplex analysis

For samples exhibiting unusual melting curves or in cases where it was difficult to differentiate the melting profiles, capillaries were opened and transferred to microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and the contents were centrifuged (Centrifuge 5415C, Eppendorf AG, Hamburg, Germany) shortly to pool the amplicons in the new tube. Five microliters of each sample were then combined with 5 µl of the amplicon of the reference HPV 16 (NCBI GenBank accession number K02718) and were subjected to rapid heating to 98°C for 1 minute then held at 40°C for 5 minutes and cooled down to 4°C in a the thermal cycler to favour heteroduplex formation (heteroduplexing). Those samples were transferred to new capillaries, melted and analyzed again as previously described.

2.5. Sequencing

Samples were purified using QIAquick PCR Purification Kit according to the manufacturer's instructions (Qiagen GmbH, Duesseldorf, Germany). Five hundred nanograms of sample were sequenced with the 16ORF5' and 16ORF3' primers at the local core sequencing facility. The BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) was used. The products were analyzed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using a corresponding Sequencing analysis 5.1 programme. The obtained sequences were compared and aligned with HPV 16 variant sequences from the NCBI database (http://www.ncbi.nlm.nih.gov) using the Bioedit programme (v 5.0.9 availble at http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

3. Results

Of the total of 255 samples, the amplicons with inner primers were successfully amplified in 239 samples. Furthermore, 4 samples had to be discarded as the melting curves were ambiguous and amplicons were found to be of poor quality after electrophoresis on the 2% agarose gel. The melting curves of the remaining 235 samples were analyzed either solely or after heteroduplexing with the known reference HPV 16 amplicon.

The melting profiles of most samples containing the common European reference variant and the most common E6-350G variant were distant enough on the difference plot to be properly distinguished, as were some other less common variants (Fig. 1). To resolve the ambiguous plots and to verify the unusual profiles, equal volume of the amplicon of the reference variant was added to the suspected variant sample. After heteroduplexing, the resulting melting profile of E6-350G and other variants were more distinct, while the addition of the reference amplicons to another sample containing the reference variant did not change the melting profile, as expected (Fig. 2).

Samples were classified into the three most common melting profile groups on the basis of their derivative curve plots. A distinct profile of the reference HPV 16 and the E6-350G HPV 16 variant was found in 75 (31.9%) and 118 (50.2%) of 235 cases, respectively, while the remaining 42 samples (17.9%) had unusual melting profiles.

All 42 samples with unusual melting profiles were sequenced as well as the additional 57 samples to validate the method. In total there were 83 Croatian samples and 16 blind

control samples from France (Table 1). Sequencing of the unusual melting profile samples, including the blind control samples, revealed 39 variant sequences, but also two samples being E6-350G HPV 16 variant and one reference HPV 16 (Table 1, superscript b).

When disregarding the samples received from France (blind control samples), the Croatian samples positive for HPV 16 (n=219) showed melting profiles to be either reference or E6-350G variant, that were found in 65 (29.7%) and 119 (54.3%) cases, respectively. All other subtypes determined by sequencing represent only a small portion (ranging from 1.2 to 8.4% each) of HPV 16 infections in Croatia (Table 1). Sequencing of the 57 samples used for the validation of the method, determined by HRM to be either reference (n=23) or the most common E6-350G (n=34), also revealed 3 discrepancies, in which additional mutations, besides the E6-350G mutation, were found (Table 1, superscript a).

The overall concordance of the findings by HRM analysis and sequencing was 93.9%, while the inter-laboratory concordance corresponding to the blind samples received from France was 100%. When taking sequencing as a standard method, the sensitivity and the specificity of the HRM analysis was calculated to be 92.9% and 94.7%, respectively.

4. Discussion

The studies of the variability of HPV relied on sequencing of the parts of the viral genome, generally of the E6 gene. A vast majority of HPV variants found in previous studies can be clustered in several major variants (Ho et al., 1993a; Ho et al., 1993b; Yamada et al., 1997), thus, sequencing every sample results in many identical sequences that are not informative. The high resolution melting analysis, on the other hand, is capable of spotting those samples that deviate from the majority and whose sequences would be informative. It is also no more complex or labour intensive to perform than a simple PCR; it does not require purification and/or cloning of the amplicons, nor enzyme digestion or gel electrophoresis for single-strand conformation polymorphism, nor any kind of hybridization with probes on a solid support. In optimal conditions, it has been demonstrated that HRM analysis has almost 100% accuracy (Reed and Wittwer, 2004) and it has previously been adapted for several microbiological applications such as rapid identification of Staphylococcus aureus genotypes and influenza subtypes (Lin et al., 2008; Stephens et al., 2008). In addition, HRM has been applied in scanning human genome DNA for several disease causing polymorphisms (reviewed in Erali et al., 2008), while at the Rudjer Boskovic Institute it has previously been

adapted to screening of polymorphism of BRCA1 and BRCA2 genes (Cvok et al., 2008).

The choice of E6 for the analysis of the variability of HPV 16 by high resolution melting relies on the fact that it is the most commonly analysed gene by sequencing, but also for it is optimal variable region. If the gene is too variable, the HRM analysis would be very complex. On the other hand, if the gene is slightly variable like E7, the HRM analysis would fail to detect different HPV variants as they often have an identical E7 region (Bernard et al., 2006).

In this study, 42 (17.9%) unusual melting profiles and 193 (82.1%) common melting profiles were identified. Thus, over 82% of resources could be saved with previous screening of samples by HRM analysis that would be spent repeatedly on sequencing of very common reference and the E6-350G HPV 16 variants. Even though there are only 4 groups of uncommon HPV 16 variants with more than 2 samples (Table 1) their melting profiles show clustering (Fig. 3), indicating that this method would be cost saving in other geographical locations where other variants with their respective melting profiles might be common.

The discrepancy between sequencing and HRM analysis is most probably due to sample quality, as these were found mostly in archival DNA samples. Those samples were extracted with the phenol-chlorophorm protocol, which might leave traces of chemicals that either inhibit PCR, interfere with dye incorporation or modify the thermal properties of the mixture and thus affect the melting profile of the sample. In support of this, it was observed that melting profiles of archival samples do not cluster as tightly as the freshly isolated DNA samples do. The concordance of sequencing and melting findings was total for the fresh cervical cancer DNA specimens, and for the blind anogenital DNA samples received from France (data not shown).

In this study, HRM analysis was adapted to evaluate the variability of HPV 16 in Croatia and found that HPV 16 is represented by 2 major subtypes, the reference and the E6-350G variant, with very few other subtypes. Samples from the harbour town Rijeka were selected specifically to find non-European HPV 16 variants, as population flow from European countries can be expected to be higher than in Zagreb due to marine traffic. However, due to a limited number of samples, only European variants were found in Rijeka.

Besides large scale studies on the variability of HPV, HRM analysis can help resolve the status of E6-350G HPV 16 variant. The role in disease progression of this particular variant is still debated, as it has been associated with greater disease progression rates and

cervical cancer in some European countries (Grodzki et al., 2006; Zehbe et al., 1998b), while not so in others (Bontkes et al., 1998; Brady et al., 1999; Nindl et al., 1999; van Duin et al., 2000; Zehbe et al., 1998a). This study demonstrated the ability of HRM analysis to differentiate this particular variant very quickly. Such an approach could be useful in finally elucidating the role of the E6-350G HPV 16 variant in cervical cancerogenesis.

The HRM analysis described herein has several benefits and applications. It is both simple and quick to perform. It has been shown to have high sensitivity and specificity. Furthermore, when used to screen samples, it can significantly reduce the cost and time required for sample sequencing in the studies on gene variability making it possible to analyse large number of samples.

Acknowledgements

The authors wish to thank all the medical assistants who have contributed to this study. The authors (I. H. and B. G.) thank Prof Harald H. Kessler for enabling the automated analysis. The authors (I. S., M. M. and M. G.) thank Jasminka Golubic Talic for her technical assistance. This study was supported by the Ministry of Science, Education and Sport of the Republic of Croatia (Grant numbers: 098-0982464-2510/2007, 098-0984364-2461 and 062-0000000-3550).

References

Bernard, H.U., Calleja-Macias, I.E. and Dunn, S.T., 2006. Genome variation of human papillomavirus types: phylogenetic and medical implications. Int J Cancer 118, 1071-6.

Bernard, H.U., Chan, S.Y., Manos, M.M., Ong, C.K., Villa, L.L., Delius, H., Peyton, C.L., Bauer, H.M. and Wheeler, C.M., 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, 1077-85.

Bontkes, H.J., van Duin, M., de Gruijl, T.D., Duggan-Keen, M.F., Walboomers, J.M., Stukart, M.J., Verheijen, R.H., Helmerhorst, T.J., Meijer, C.J., Scheper, R.J., Stevens, F.R., Dyer, P.A., Sinnott, P. and Stern, P.L., 1998. HPV 16 infection and progression of cervical intra-epithelial neoplasia: analysis of HLA polymorphism and HPV 16 E6 sequence variants. Int J Cancer 78, 166-71.

Brady, C.S., Duggan-Keen, M.F., Davidson, J.A., Varley, J.M. and Stern, P.L., 1999. Human papillomavirus type 16 E6 variants in cervical carcinoma: relationship to host genetic factors and clinical parameters. J Gen Virol 80 (Pt 12), 3233-40.

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

Cvok, M.L., Cretnik, M., Musani, V., Ozretic, P. and Levanat, S., 2008. New sequence variants in BRCA1 and BRCA2 genes detected by high-resolution melting analysis in an elderly healthy female population in Croatia. Clin Chem Lab Med 46, 1376-83.

de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U. and zur Hausen, H., 2004. Classification of papillomaviruses. Virology 324, 17-27.

Erali, M., Voelkerding, K.V. and Wittwer, C.T., 2008. High resolution melting applications for clinical laboratory medicine. Exp Mol Pathol 85, 50-8.

Grce, M., Husnjak, K., Bozikov, J., Magdic, L., Zlacki, M., Lukac, J., Fistonic, I., Sikanic-Dugic, N. and Pavelic, K., 2001. Evaluation of genital human papillomavirus infections by polymerase chain reaction among Croatian women. Anticancer Res 21, 579-84.

Grodzki, M., Besson, G., Clavel, C., Arslan, A., Franceschi, S., Birembaut, P., Tommasino, M. and 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, 820-2.

Ho, L., Chan, S.Y., Burk, R.D., Das, B.C., Fujinaga, K., Icenogle, J.P., Kahn, T., Kiviat, N., Lancaster, W., Mavromara-Nazos, P. and et al., 1993a. The genetic drift of human papillomavirus type 16 is a means of reconstructing prehistoric viral spread and the movement of ancient human populations. J Virol 67, 6413-23.

Ho, L., Tay, S.K., Chan, S.Y. and Bernard, H.U., 1993b. Sequence variants of human papillomavirus type 16 from couples suggest sexual transmission with low infectivity and polyclonality in genital neoplasia. J Infect Dis 168, 803-9.

IARC, 2007. Human Papillomaviruses Vol. 90. International Agency for Research on Cancer (IARC), World Health Organization.

Lin, J.H., Tseng, C.P., Chen, Y.J., Lin, C.Y., Chang, S.S., Wu, H.S. and Cheng, J.C., 2008. Rapid differentiation of influenza A virus subtypes and genetic screening for virus variants by high-resolution melting analysis. J Clin Microbiol 46, 1090-7.

Ljubojevic, N., Babic, S., Audy-Jurkovic, S., Ovanin-Rakic, A., Jukic, S., Babic, D., Grubisic, G., Radakovic, B. and Ljubojevic-Grgec, D., 2001. Improved national Croatian diagnostic and therapeutic guidelines for premalignant lesions of the uterine cervix with some cost-benefit aspects. Coll Antropol 25, 467-74.

Milutin-Gasperov, N., Sabol, I., Halec, G., Matovina, M. and Grce, M., 2007. Retrospective study of the prevalence of high-risk human papillomaviruses among Croatian women. Coll Antropol 31 Suppl 2, 89-96.

Munoz, N., Bosch, F.X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K.V., Snijders, P.J. and Meijer, C.J., 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348, 518-27.

Nindl, I., Rindfleisch, K., Lotz, B., Schneider, A. and Durst, M., 1999. Uniform distribution of HPV 16 E6 and E7 variants in patients with normal histology, cervical intra-epithelial neoplasia and cervical cancer. Int J Cancer 82, 203-7.

Ortiz, M., Torres, M., Munoz, L., Fernandez-Garcia, E., Canals, J., Cabornero, A.I., Aguilar, E., Ballesteros, J., Del Amo, J. and Garcia-Saiz, A., 2006. Oncogenic human papillomavirus (HPV) type distribution and HPV 16 E6 variants in two Spanish population groups with different levels of HPV infection risk. J Clin Microbiol 44, 1428-34.

Reed, G.H. and Wittwer, C.T., 2004. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clin Chem 50, 1748-54.

Sotlar, K., Diemer, D., Dethleffs, A., Hack, Y., Stubner, A., Vollmer, N., Menton, S., Menton, M., Dietz, K., Wallwiener, D., Kandolf, R. and Bultmann, B., 2004. Detection and typing of human papillomavirus by e6 nested multiplex PCR. J Clin Microbiol 42, 3176-84.

Si-Mohamed, A., Ndjoyi-Mbiguino, A,, Cuschieri, K., Onas, I.N., Colombet, I., Ozouaki, F., Goff, J.L., Cubie, H., Bélec, L., 2005. High prevalence of high-risk oncogenic human papillomaviruses harboring atypical distribution in women of childbearing age living in Libreville, Gabon. J Med Virol 77, 430-8.

Stephens, A.J., Inman-Bamber, J., Giffard, P.M. and Huygens, F., 2008. High-resolution melting analysis of the spa repeat region of Staphylococcus aureus. Clin Chem 54, 432-6.

van Duin, M., Snijders, P.J., Vossen, M.T., Klaassen, E., Voorhorst, F., Verheijen, R.H., Helmerhorst, T.J., Meijer, C.J. and Walboomers, J.M., 2000. Analysis of human papillomavirus type 16 E6 variants in relation to p53 codon 72 polymorphism genotypes in cervical carcinogenesis. J Gen Virol 81, 317-25.

Wittwer, C.T., Reed, G.H., Gundry, C.N., Vandersteen, J.G. and Pryor, R.J., 2003. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49, 853-60.

Yamada, T., Manos, M.M., Peto, J., Greer, C.E., Munoz, N., Bosch, F.X. and Wheeler, C.M., 1997. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective. J Virol 71, 2463-72.

Zehbe, I., Voglino, G., Delius, H., Wilander, E. and Tommasino, M., 1998a. Risk of cervical cancer and geographical variations of human papillomavirus 16 E6 polymorphisms. Lancet 352, 1441-2.

Zehbe, I., Wilander, E., Delius, H. and Tommasino, M., 1998b. Human papillomavirus 16 E6 variants are more prevalent in invasive cervical carcinoma than the prototype. Cancer Res 58, 829-33.

Figure 1. Melting curves of representative samples containing A) the reference variant and the E6 350G variant of HPV 16 showing clustering of these melting profiles, and B) the same samples shown with other variants; the melting curves are presented by the temperature (T; ^{o}C) and rate of fluorescence change with temperature (first derivative plot fluorescence vs. temperature; -dF/dT); two distinct melting patterns are visible, one corresponding to the reference variant and the other to the E6-350G variant, while other less common variants have clearly different patterns

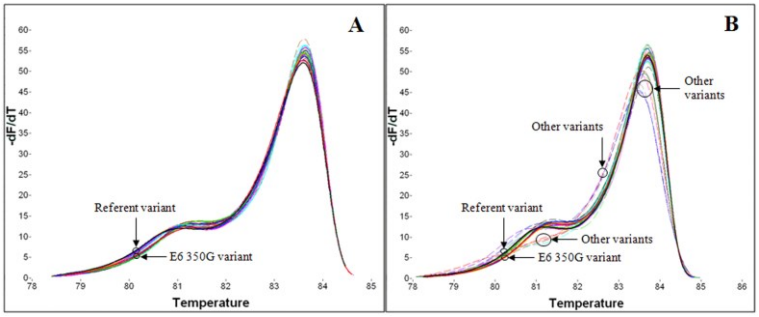

Figure 2. Melting curves of representative samples containing A) the reference variant and the E6 350G variant that was previously combined and heteroduplexed with the reference variant and B) the same samples shown with other variants; the melting curves are presented by the temperature (T; ^{o}C) and rate of fluorescence change with temperature (first derivative plot fluorescence vs. temperature; -dF/dT); heteroduplexing allowed better discrimination of different patterns

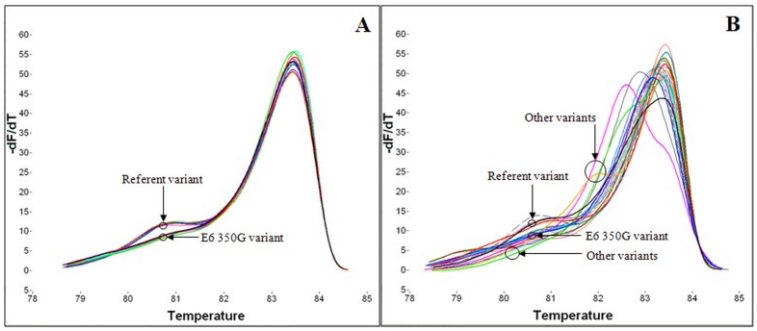

Figure 3. Melting profiles of samples with the same mutations determined by sequencing presented by A) the first derivative plot of fluorescence vs. temperature (-dF/dT) and the temperature (T; ºC) and B) the fluorescence difference plot; less common variants containing the same mutations have very similar melting patterns as can be seen in both representations of melting plots

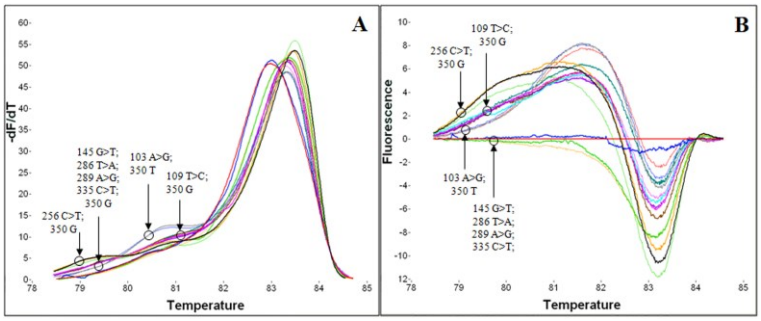

Table 1. Variability of the E6 gene of HPV 16 assessed by sequencing

	Sequence (GenBank accession number)	Number of cases	Melting errors	Percent in population	Nucleotide position 83	93	94	103	105	109	119	131	132	143	145	154	158	173	176	182	212	246	256	286	289	335	350	400	403	412	433	456	533	546	Similar to
Referent sequences	Reference variant (K02718)				a	a	g	a	t	t	t	a	g	c	g	a	c	c	g	a	t	g	c	t	a	c	t	g	a	t	g	a	a	a	Reference
	African T1 (AF536180)				c								c	g	t									a	g	t									African T1
	African T2 (AF472509)									c			t	g	t									a	g	t			g						African T2
	Asian American (AF402678)														t									a	g	t	g				a				Asian American
Blind control samples	083 A>C; 132 G>C; 145 G>T; 286 T>A; 289 A>G; 335 C>	1	0		c									c		t									g	t									African T1
	119 T>C; 350 T>G;	1	0								c																g								E6-350G
	131 A>G; 350 T>G;	1	0									a															g								E6-350G
	143 C>G; 145 G>T; 286 T>A; 289 A>G; 335 C>T;	1	0											g	t									a	g	t									African
	145 G>T; 286 T>A; 289 A>G; 335 C>T; 350 T>G;	1	0												t									a	g	t	g								Asian American
	176 G>A;	1	0																a																Reference
	212 T>C;	1	0																		c														Reference
	350 T>G;	5	0																								g								E6-350G
	Reference variant	4	0																																Reference
	Total	16	0																																
Croatian samples	093 A>T; 145 G>T; 286 T>A; 289 A>G; 335 C>T; 350	1	0	1.2%		t									t									a		t	g								Asian American
	094 G>A; 350 T>G;	1	0	1.2%			a																				g								E6-350G
	103 A>G;	3	0	3.6%				g																											Reference
	105 T>C; 350 T>G; 456 A>G;	1	0	1.2%					c																		g					g			E6-350G
	109 T>C; 350 T>G;	7	0	8.4%						c																	g								E6-350G
	131 A>G; 350 T>G;	1	0	1.2%								g															g								E6-350G
	131 A>G;	1	0	1.2%								g																							Reference
	132 G>C; 143 C>G; 145 G>T; 286 T>A; 289 A>G; 335	1	0	1.2%									c	g	t										g	t									African T1
	145 G>T; 286 T>A; 289 A>G; 335 C>T; 350 T>G;	4	0	4.8%											t									a	g		g								Asian American
	154 A>G; 350 T>G;	2	0	2.4%												g											g								E6-350G
	158 C>T;	1	0	1.2%													t																		Reference
	173 C>T;	1	0	1.2%														t																	Reference
	176 G>C; 350 T>G;	1	1[a]	1.2%															c								g								E6-350G
	182 A>C; 350 T>G;	1	1[a]	1.2%																c							g								E6-350G
	246 G>T; 350 T>G;	1	0	1.2%																		t					g								E6-350G
	256 C>T; 350 T>G;	3	0	3.6%																			t				g								E6-350G
	256 C>T; 350 T>G; 546 A>G;	1	0	1.2%																			t				g							g	E6-350G
	350 T>G; 533 A>C;	1	0	1.2%																							g					c			E6-350G
	350 T>G; 412 T>C;	2	1[a]	2.4%																							g			c					E6-350G
	400 G>A;	1	0	1.2%																								a							Reference
	350 T>G;	27	2[b]	32.5%																							g								E6-350G
	Reference variant	21	1[b]	25.3%																															Reference
	Total	83	6	100.0%																															

[a] melting indicated E6-350G variant profile; [b] melting result indicated unknown pattern