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# Investigation of human papillomavirus type 16 prototypes and variants in cervical cancer patients

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Cervical cancer is the second most common cancer in women worldwide and the fourth in Lithuania. Infection with human papillomavirus (HPV) 16 is an important risk factor associated with cervical cancer; more than 50% of cervical cancer tissues have DNA of HPV16. Intratypic variants have been reported; although they differ in prevalence, biological and biochemical properties, their implication in the etiology of cervical cancer is still uncertain. The objective of this study was to identify the distribution of HPV16 E6 variants among women with invasive cervical cancer and healthy women and to assess an association of HPV16 E6 variants with cervical cancer risk. 111 HPV16 positive samples were analyzed: 88 were from women with invasive cervical cancer, 23 from healthy women volunteers without cancer. 524 base pairs from the HPV16 E6 gene were amplified by PCR and the variant status subsequently determined by DNA sequencing. The European L83V variant was detected most frequently in the samples of both cervical cancer and control group women (64.8 and 73.9% respectively). The prototype was detected in 22.7% of cervical cancer and in 17.4% of control samples. The remaining 12.5% in the cervical cancer group and 8.7% in control were referred to as multivariants. In conclusion, women infected with the European L83V variant are not at a higher risk of developing cervical cancer (OR=0.67, 95% CI 0.15–2.42) in comparison with those infected with the HPV16 prototype.

**Key words:** HPV, cervical cancer, HPV16 E6 variants

## INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide and the fourth in Lithuania. Infection with human papillomaviruses (HPVs) leads to hyperproliferative lesions in humans at specific anatomical sites which are determined by the tropism of individual viral genotypes. Among the more than 100 types of HPVs identified to date, a subset of mucosotropic viruses which infect the epithelial lining of the anogenital tract and oral cavity are causally associated with the great majority of cervical cancers worldwide [1]. Approximately 30 of HPV types have been associated with cervical neoplasia [2–4]. Certain types of HPV, including types 16, 18, 31, 33, 35, 45, 52, 56 and 58, play a major role in the carcinogenesis of cervical cancer [5, 6]. In fact, the HPV viral DNA is identified in at least 90% of cervical carcinomas by PCR [7, 8]. The HPV type 16 is most often linked with invasive cervical cancers (ICC) and is detected in approximately 50% of specimens from patients with ICC [7]. HPVs vary genetically not only among but also within the types. Intratypic variants are defined as HPVs that vary by 2% or less in specified regions of the genome [9].

Numerous variants of HPV16 have been identified in different geographic locations and ethnic groups [10, 11]. Previous studies inferred five distinct phylogenetic branches among HPV16 variants: European (E), Asian (As), Asian–American (As-Am), African-1 (Af-1), and African-2 (Af-2), corresponding to the geographic locations from which the samples were obtained. The originally identified reference sequence, the so-called prototype, belongs to the European subtype [12, 13]. These can be further divided into multiple genotypes. Polymorphism in the E6 gene correlates with the severity of lesions in several European populations [14]. Subsequent studies by sequence analyses of the HPV16 variants in other genomic regions (e.g., E6, L2, and L1) expanded and complemented this phylogenetic hypothesis [15]. The European variant of HPV16 has been shown recently to be more prevalent in invasive cervical carcinoma than in the preinvasive lesions or normal cervix.

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This HPV16 variant possesses a common mutation (T to G) in nucleotide 350 (codon 83) of the E6 gene, resulting in an amino acid shift, L83V, in the E6 protein. This mutation was believed to signify preinvasive cervical lesions with a high probability of progression to invasive carcinoma [16]. Given its prevalence in cervical carcinoma, HPV16 is the focus of most prophylactic and therapeutic vaccine development efforts that are under way. Intratype HPV16 amino acid variations may be relevant to the generation of specific immunoresponses [17].

Studies on the prevalence of HPV, its types and variants in cervical carcinoma patients and healthy women were conducted in Lithuania [18, 19]. HPV16 is the most prevalent type in cervical cancer patients and in healthy women. Whereas HPV16 and its variants are the focus of most prophylactic and therapeutic vaccine development, the objective of this study was to identify the distribution of HPV16 E6 variants among Lithuanian women with invasive cervical cancer and healthy women and to assess the association of HPV16 E6 variants with cervical cancer risk.

## MATERIALS AND METHODS

*Study materials.* 111 samples positive for HPV16 were investigated: 88 samples from cervical cancer patients and 23 from healthy women without cancer. Brush swabs were used to obtain the cervical samples. Cellular material from the brush was collected in 1 ml of transport medium (Digene diagnostics). The cells were stored at –20 °C until HPV DNA testing. Cervical cancer samples were collected at the Institute of Oncology, Vilnius University (Vilnius, Lithuania).

*HPV16 typing.* HPV type 16 was identified using type-specific polymerase chain reaction (TS-PCR) [20]. HPV16 detection was performed at the Department of Molecular Pathology, St. Elisabeth Hospital (Herentals, Belgium). HPV16 E6 variants were detected using sequencing. 82 samples (73 from cervical cancer, 9 from control) were sequenced at the Invitek Sequencing Centre (Berlin, Germany). The remaining 29 samples were sequenced at the Institute of Biotechnology DNA Sequencing Core (Vilnius, Lithuania).

HPV DNA was purified using a Genomic DNA Purification Kit (Fermentas, Lithuania). PCR for HPV16 detection was performed in 50 µl of PCR solution containing a dNTP Mix consisting of 2 mM of each dATP, dCTP, dGTP and dTTP supplemented with 10 × PCR buffer (100 mM Tris-HCl (pH 8.8), 500 mM KCl 0.8% Nonidet P40 and 15 mM $MgCl_2$), 50 pmol of each primer and 0.5 units Taq polymerase (Fermentas, Lithuania). Using a PCR processor (Eppendorff personal cycler, Germany), PCR was performed for 40 cycles. Each cycle consisted of a denaturation step at 95 °C for 1 min, primer annealing step at 55 °C for 1 min 30, and chain elongation step at 72 °C for 1 min. A final extension of 10 minutes at 72 °C was performed. Each PCR experiment was performed with positive and negative PCR controls. As a positive control, DNA from HeLa cells was used. Negative control samples contained no DNA. As a control for DNA integrity, all samples were tested for the β globine gene. PCR products were analysed by electrophoresis in 2% agarose gel stained with ethidium bromide.

*Detection of HPV16 E6 variants.* All 111 samples were analyzed for HPV16 E6 gene variants. The E6 ORF primers were 5'-CGAAACCGGTTAGTATAA-3' and 5'-GTATCTCCATGCATGATT-3' [11], spaning nt 52-575. Target sequences were amplified in a 50 µl reaction volume using the same amplification protocol as described earlier.

For HPV16 DNA sequencing, PCR products were extracted from the agarosis gel using DNA Extraction Kit (Fermentas, Lithuania). DNA sequencing was performed with BigDye® Terminator v3.1 Cycle Sequencing Kit.

*Statistical analysis.* To estimate the risk of cervical cancer associated with HPV16 E6 variants, odds ratios (OR) and 95% confidence intervals (CI) were calculated, the logistic regression model was used.

## RESULTS AND DISCUSSION

In this study, HPV16 E6 intratypic variations were investigated. Table 1 presents the distribution of HPV16 E6 prototypes and variants. The European L83V variant was detected most frequently in both cervical cancer and control samples (64.8 and 73.9%, respectively). Further, in 19 of cervical cancer samples additional nucleotide changes together with mutation at 350 position were found. A nucleotide change at 109 position, when T is changed by C, together with 350G mutation was highly frequent (7 cervical cancer and 4 control samples). In 2 samples from cervical cancer patients, additional mutation in 118 position was detected (A was changed by G). In the control group, additional nucleotide changes together with 350G mutation were found in 6 samples of the L83V variant.

The prototype was detected in 22.7% of cervical cancer and in 17.4% of control samples. The remaining 12.5% in the study group and 8.7% in control were referred to as multivariants. In these samples one or more nucleotide changes, without change at position 350, were detected. In Table 2 we show all nucleotide changes in the HPV16 E6 region in cervical cancer and control samples. We have found that women infected with the European L83V

Table 1. **Distribution of HPV16 prototypes and variants in cervical cancer and control samples and association with cervical cancer risk**

| HPV 16 | Cervical cancer samples | | Control samples | | OR (95%CI) |
|---|---|---|---|---|---|
| | n | % | n | % | |
| Prototype | 20 | 22.7 | 4 | 17.4 | 1 (ref) |
| L83V variant | 57 | 64.8 | 17 | 73.9 | 0.67 (0.15–2.42) |
| Multi-variant | 11 | 12.5 | 2 | 8.7 | 1.1 (0.1314.00) |

Table 2. **HPV16 E6 gene nucleotide changes in cervical cancer and control samples**

| Nucleotide position in HPV16 E6 seguence | Variants | Number |
|---|---|---|
| *Cervical cancer samples* | | |
| *350G sequences* | | |
| **350G** | L83V | 37 |
| 109C, **350G** | L83V | 7 |
| 118G, **350G** | L83V | 2 |
| 145G, 286A, 289G, 335T, **350G**, 532G | L83V | 1 |
| 145G, 286A, 289G, 335T, **350G** | L83V | 1 |
| 109C, 112A, 120C, **350G** | L83V | 1 |
| 103C, **350G**, 542G | L83V | 1 |
| 133T, **350G** | L83V | 1 |
| 173T, **350G** | L83V | 1 |
| 176A, **350G** | L83V | 1 |
| 252T, **350G** | L83V | 1 |
| 286T, **350G** | L83V | 1 |
| 310G, **350G** | L83V | 1 |
| **350G**, 376C | L83V | 1 |
| *Cervical cancer samples* | | |
| *350T sequences* | | |
| **350T** | Prototype | 20 |
| 094C, **350T**, 525T, 558T | Multivariant | 1 |
| 142C, 188A, **350T** | Multivariant | 1 |
| 193G, 224A, **350T** | Multivariant | 1 |
| 091G, **350T** | Multivariant | 1 |
| 109C, **350T** | Multivariant | 1 |
| 114T, **350T** | Multivariant | 1 |
| 131G, **350T** | Multivariant | 1 |
| 252T, **350T** | Multivariant | 1 |
| 253T, **350T** | Multivariant | 1 |
| 256T, **350T** | Multivariant | 1 |
| **350T**, 525T | Multivariant | 1 |
| *Control samples* | | |
| *350G sequences* | | |
| **350G** | L83V | 11 |
| 109C, **350G** | L83V | 3 |
| 110T, 139G, 222T, **350G**, 542G | L83V | 1 |
| 132C, 194G, 222T, **350G**, 542G | L83V | 1 |
| **350G**, 567G, 569A | L83V | 1 |
| *Control samples* | | |
| *350T sequences* | | |
| **350T** | Prototype | 4 |
| 110T, 111T, 205A, 223A, 272C, 274A, **350T** | Multivariant | 1 |
| 132C, 139G, 222T, **350T** | Multivariant | 1 |

variant are not at a higher risk of developing cervical cancer (OR = 0.67, 95% CI 0.15–2.42) in comparison to those infected with the HPV16 prototype.

These data show that the HPV16 L83V variant was detected more frequently than the prototype. However, both the HPV16 prototype and L83V variant tended to be represented with a similar frequency in cervical cancer and normal (without cancer) cervical scrape samples. Zehbe et al. [11] found that in the Swedish population cervical carcinomas were almost exclusively (in 94%) associated with HPV16 variants. In this study, authors assessed an association of HPV16 L83V variants with a higher risk of cervical cancer development. Yamada et al. [12] investigated cervical carcinoma samples from Germany, Poland and Spain populations and revealed that the HPV16 prototype was present in 34% of these samples. Our data from these studies. We did not find an association of HPV16 L83V variants with a higher risk of cervical cancer development. However, the observed differences may reflect a population-dependent oncogenicity of the HPV16 L83V variant [20, 21].

It is well known that in cervical carcinogenesis other risk factors are involved. Likewise, the histological structure of cancer may play an important role. A strong link between HPV and cancer is observed for both squamous (i.e., SCC) and glandular (e.g., adenocarcinomas and adenosquamous

carcinomas) forms of the disease. However, while a HPV infection appears to be required for the development of both SCC and AC, the distribution of HPV types seen in these two forms of the disease differ. Furthermore, studies have suggested that the cofactors associated with the development of SCC and AC are different. In addition to HPV infection, factors such as cigarette smoking and multigravidity are associated with an increased risk of SCC, whereas these same exposures are associated with an decreased risk of AC, pointing to differences in the biological mechanisms through which these different tumor types arise. It has been recently suggested that intratypic variants of HPV might confer differential risk of cervical disease. However, whether the distributions of intratypic HPV variants seen in these two histological forms of cervical disease differ is unknown [22]. These primary data need further large-scale, epidemiologically sound studies to elucidate the possible relationships between HPV16 E6 variants, the histological structure of cancer, precancerous lesions and other risk factors.

## CONCLUSION

The HPV16 L83V variant was detected more frequently than its prototype in samples from both cervical cancer and control groups. According to our data, women infected with the European L83V variant are not at a higher risk of developing cervical cancer (OR = 0.67, 95% CI 0.15–2.42) in comparison to those infected with HPV16 prototype.

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## GIMDOS KAKLELIO VĖŽIU SERGANČIŲ MOTERŲ 16-O TIPO ŽMOGAUS PAPILOMOS VIRUSO PROTOTIPŲ IR VARIANTŲ TYRIMAI

Santrauka

Sergamumas gimdos kaklelio vėžiu užima antrą vietą tarp moterų piktybinių navikų pasaulyje ir ketvirtą vietą Lietuvoje. 16-o tipo žmogaus papilomos viruso (ŽPV16) sukelta infekcija yra vienas svarbiausių rizikos veiksnių vystantis šiai patologijai: daugiau kaip 50% šių navikų yra nustatoma specifinė ŽPV16 DNR. Egzistuoja įvairūs šio tipo viruso variantai, besiskiriantys paplitimu, biologinėmis ir biocheminėmis savybėmis, tačiau jų poveikis vystantis gimdos kaklelio vėžiui vis dar lieka neaiškus ir toliau tyrinėjamas. Šio tyrimo tikslas – ištirti ŽPV16 E6 prototipų bei variantų pasiskirstymą gimdos kaklelio vėžiu sergančių ir sveikų moterų ląstelių mėginiuose, gautuose nubraukus ląsteles nuo gimdos kaklelio paviršinio sluoksnio, ir nustatyti galimą gimdos kaklelio vėžio riziką, siejamą su Europiniu L83V variantu. Tyrimui buvo atrinkta 111 ŽPV16 tipo virusu infekuotų gimdos kaklelio mėginių: 88 mėginiai gauti iš sergančių gimdos kaklelio vėžiu moterų, 23 – iš sveikų be vėžinės patologijos moterų. 524 bp ilgio ŽPV16 E6 geno produktas buvo gautas amplifikacijos būdu atliekant polimerazės grandininę reakciją, prototipas ir įvairūs variantai patikslinti sekvenuojant amplifikuotą DNR. Dažniausiai mūsų tirtosioms moterims, ir gimdos kaklelio vėžio, ir kontrolinėje grupėse, buvo identifikuotas europinis L83V variantas (atitinkamai 64,8 ir 73,9%.). ŽPV16 E6 prototipas buvo identifikuotas 22,7% vėžiu sergančioms moterims, tarp sveikų moterų – 17,4%. Likusiuose mėginiuose buvo identifikuoti vadinamieji multivariantai (12,5% gimdos kaklelio vėžio ir 8,7% mėginiuose, paimtuose iš sveikų gimdos kaklelio audinių). Mūsų tyrimo duomenimis, moterys, infekuotos europiniu L83V variantu, neturi didesnės rizikos susirgti gimdos kaklelio vėžiu negu infekuotos ŽPV16 prototipu (ŠS = 0,67, 95% PI 0,15–2,42).