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# Distribution of human papillomavirus type 16 variants in Lithuanian women with cervical cancer

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ABSTRACT

*Background and objective:* Cervical cancer usually is caused by HPV 16. However, HPV 16 varies within type; different genotypes are described as prototype or variants. Prevalence of different variants differ according the geographic regions and has an unequal impact for cervical cancer development. Our study aimed to identify which variant of HPV 16 was most prevalent in biological samples taken from Lithuanian women with cervical cancer.

*Materials and methods:* A total of 122 HPV 16 positive cervical samples (invasive cancer and cervical intraepithelial neoplasia) were investigated and sequenced to identify different variants. HPV 16 was detected using type specific PCR, exact sequence of the virus was obtained by viral DNA sequencing.

*Results:* Adequate HPV sequence was detected in 106 cases from 122 (86.9% of all cases). After histological confirmation, 96 cases were included in the final analysis. In 33 cases (34.4%) HPV 16 prototype was detected; in 50 cases (52.1%), L83V variant; and in remaining 13 cases (13.5%), multivariant of HPV 16. The frequency of L83V variant in invasive cancer and carcinoma *in situ* samples was the same (66.7% and 62.0%, respectively; $P = 0.696$). Of analyzed multivariants, 10 were attributed to the European phylogenetic line; 1, to the North American, and 1, to the Asian-American. One sample was not attributed to any of the known phylogenetic lines.

*Conclusions:* The European HPV 16 L83V variant is usually associated with high risk of cervical cancer among women. However, statistically significant difference was not achieved when

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comparing difference of L83V variants between investigated groups and in HPV 16 L83V variant and prototype distribution in CIN3/Ca *in situ* and cancer.



## 1. Introduction

Cervical cancer is the most common cancer in women not only in Lithuania, but also in many countries around the world. One of the main risk factors for cervical intraepithelial lesions and cervical cancer development is the human papilloma virus (HPV) infection [1].

According to the literature more than 120 different HPV genotypes are identified to date. Viral classification is based on the differences in sequences of E6, E7 and/or L1 viral genes [1]. Different types of HPV are separated with detection of limited nucleotide changes in the coding (<2%) and non-coding (5%) regions [2]. Viruses of one separate type are characterized by the similarity or homology of the viral sequence at least 90% with prototypic variant. Differences up to 2% are defined as variants [3].

Despite such a large genotypic and phenotypic similarity of different HPV types, biological and clinical significance of different HPV varies. For example, about 40 of all currently known HPV types infect the genital mucosa and skin. Fifteen types of viruses are specific for cervical cancer. Moreover, HPV 18 and 16 are the most common infectors of cervical epithelium and are found in more than 70% of all cancers of cervicis uteri [4]. However, different HPV types have different oncogenic potential, accordingly this potential traditionally viruses are divided into high, medium and low-risk types [1]. As stated earlier, cervical pathology occurs usually after longitudinal persistence of oncogenic types HPV 16 or 18. However, not all women infected by these viruses develop cervical cancer. Different variants of HPV 16 exist with different prevalence according geographic regions and uneven oncogenicity: some of them are often found in cervical cancer, others in precancerous or normal epithelium [1,5,6].

Mutations in HPV genome occur very slowly because these viruses have double-stranded DNA. DNA viruses replicate using host cell DNA polymerases which could detect and repair the replication errors. Moreover, viruses evolve together with the population [5,6]. Nevertheless, the nucleotide polymorphism could lead to rare random mutations. These genetic changes are observed in the case of HPV 16.

An HPV genetic variant is defined as the viral genome characterized by a unique single nucleotide polymorphism (SNPs) combination. The new variant could be separated if the viral genome differs in less than 1% in their nucleotide sequence [1]. Several studies based on E6 gene and/or LCR of European and American women sequencing samples indicate that HPV variants have a different impact to the persistence of the virus and cervical cancer development. After a detailed analysis of 985 HPV 16-infected individuals from 29 countries, more than 49 variable nucleotides in the E6 gene (from 104 to 559 nt), which were located in 68 different positions, and 169 nucleotides in the variable region of LCR (from 7157 to 83 nt), located in 288 different positions, were identified. Together, these formed 353 unique options that have been carried out in the phylogenetic analysis in order to classify them into the general geographical lines. Two major categories of HPV16 variants have been defined: European (EUR) and non-European (NE) [7]. Non-European variants are classified into African (AFR-1 and AFR-2), Asian (As), Asian-American (AA) and North American (NA) [6–8]. The most commonly detected single-nucleotide polymorphism in the European line is T350G mutation in the E6 gene leading to an amino acid change of leucine by valine (or named L83V variant) [5,6]. This SNP can be found individually or together with other mutations or polymorphisms. Several studies were carried out to investigate an impact of different polymorphic variants on cancer development; however, there is no strong evidence to use some specific types as biological or phylogenetic markers. Despite the lack of evidence for biological behavior of viruses, several combinations of SNPs have already given us additional phylogenetic information on virus evolution [6]. Different authors conclude that the number of genetic variants within HPV 16 differing in their geographical origins, ethnicity, relationship with cervical cancer, viral persistence features and cervical changes in clinical course [5–8].

On the other hand it is important to stress that different variants of the separate phylogenetic lines are distributed unevenly in the world [9]. Genetic variants belonging to the AFR lines mostly found in Africa, which shows the isolation of the African population. Meanwhile, the EUR line genetic variants are prevailed widely in all continents [6].

Our study aimed at identifying which of HPV 16 variants was most common in biological samples taken from Lithuanian women with cervical cancer and high-grade cervical intraepithelial neoplasia.

## 2. Material and methods

### 2.1. *Collection of cervical samples*

The analysis of HPV 16 variants was performed on the HPV 16 positive cervical samples from the previously conducted HPV prevalence study [10,11]. The study protocol and informed consent were approved by the Vilnius Regional Committee of Bioethics (June 3, 2009, No. 158200-6-062-16). All cervical samples were taken from women attending to the Outpatients Clinic of National Cancer Institute with suspicion of invasive cervical cancer or high-grade cervical intraepithelial neoplasia (CIN3/Carcinoma *in situ*). Final diagnosis was confirmed by histology. DNA extracted from cervical cells was used for HPV

investigation. HPV screening and testing was performed using standard protocols developed in the previous study [12].

### 2.2. Detection of HPV16 genetical variants

Definition and analysis of HPV 16 genetic variants were carried out in several stages. Firstly, HPV 16 DNA was amplified using common HPV 16 E6 gene specific primers. The primers sequences are shown in Table 1.

PCR was carried out in 50 μL reaction volume using *DreamTaq*™ *Green PCR Master Mix* (Thermo Fisher Scientific, Lithuania). The PCR mix consisted of 10 μL DNA and 40 μL HPV Master Mix containing 5 μL sense and antisense primers (*Oligo 1* and *Oligo 2*) (Table 1), 4 μL nuclease-free water, and 26 μL *DreamTaq*™ *Green PCR Master Mix*.

PCR was performed using a thermal cycler (SensoQuest labcycler, Germany). The PCR cycle for HPV 16 E6 gene amplification was performed in the following steps: initial denaturation cycle at 95 °C temperature for 4 min; 40 cycles of PCR product amplification with each cycle consisting of 1-min denaturation step at 95 °C, 2-min annealing at 58 °C, 1.5-min extension at 72 °C; and 1 cycle of elongation at 72 °C temperature for 7 min.

After amplification all PCR products were analyzed using electrophoresis. Electrophoresis was carried out in 1% agarose gel, the ingredients of which were: 1% Top Vision™ LE GQ agarose (Thermo Fisher Scientific, Lithuania), 100 mL of TAE buffer (Thermo Fisher Scientific, Lithuania), the Atlas Sight DNA Stain (Bioatlas, Estonia), which is a non-cancerogenic and alternative dye for the ethidium bromide. As standard marker for PCR product DNA length measuring MassRuler™ DNA Ladder, Low Range (Thermo Fisher Scientific, Lithuania) was used. Amplified PCR product DNA concentration was analyzed under 350 nm UV light source in transilluminator (Herolab, Germany).

The second step of DNA preparation for target fragment sequencing was cutting and purification of DNA fragments from the agarose gel. GeneJET™ Gel extraction Kit (Thermo Fisher Scientific, Lithuania) was used for DNA extraction from the gel. DNA was purified according to the manufacturer's protocol. Finally, purified products were sequenced using the same HPV16 E6 gene primers, sequencing was performed in the Sequencing Center, Institute of Biotechnology, Vilnius University.

### 2.3. Statistical analysis

After sequencing nucleotide sequences were aligned and compared with known HPV 16 sequences freely available in GenBank database BLAST software (http://www.ncbi.nih.gov/blast). The results were analyzed using the CLC program. HPV16 E6 proteins dimensional structures were visualized and constructed using PYMOL program. The distribution of HPV 16 variants was described using descriptive statistics; differences between investigated groups were estimated using MedCalc program. The chi-square test was used for the comparison between proportions. Results were statistically significant if $P < 0.05$.

**Table 1 – Primers sequences for the HPV type 16 E6 gene fragments amplification and sequencing.**

| Primer | Sequence (5′–3′) | Amplified product (bp) |
|---|---|---|
| Oligo 1 | 5′-CGAAACCGGTGTATTAA-3′ | 524 bp |
| Oligo 2 | 5′-GTATCTCCATATGATT-3′ | 524 bp |

## 3. Results

A total of 122 HPV 16 positive cervical samples were obtained for the analysis of viral genetic variants and sequenced. During the sequencing, an adequate signal was detected from 106 samples (86.9% of all sequenced samples); 16 samples failed probably due to insufficient quantity of extracted DNA. Additional 10 samples were excluded from the final analysis because cytological changes were not confirmed by histology (histological analysis was not performed). Primary analysis of sequenced DNA showed that in mostly samples European L83V variant of HPV16 was detected: this variant was detected in 50 samples (52.1%). In 33 samples (34.4%) the prototype of HPV 16 was found and in remaining 13 samples (13.5%) multivariants were detected (Table 2). Fig. 1 shows the dimensional structure of HPV 16 E6 protein.

### 3.1. HPV 16 E6 variants distribution according histological diagnosis

HPV 16 prototypes and L83V variants (excluding 13 cases of multivariants) distribution was analyzed according to histological diagnosis. After histological evaluation 25 cases were diagnosed with invasive cancer: 24 cases with squamous cell carcinoma (SCC) and 1 with adenocarcinoma (AD). In 50 cases with cervical intraepithelial neoplasia grade 3 or carcinoma *in situ* (CIN3/Ca *in situ*) was confirmed. In 5 cases cervical intraepithelial neoplasia grade 1 (CIN1), 2 (CIN2) or no intraepithelial changes (NORMA) were stated. Our analysis showed that the L83V variant was most frequently detected in investigated samples: it was found in 16 cases (66.7%) of SCC and 31 cases (62.0%) of CIN3/Ca *in situ* (Table 3). Moreover, L83V frequency in cervical cancer and CIN3/Ca *in situ* cases is similar: no statistically significant difference was stated when comparing HPV 16 prototype and L83V variant distribution in both groups of tested samples ($P = 0.696$ when comparing difference of L83V variants between SCC and CIN3/Ca *in situ*).

**Table 2 – Distribution of HPV 16 E6 prototype and variants in cervical samples.**

| HPV16 E6 variants | Cervical samples | |
|---|---|---|
| | *n* | % |
| Prototype | 33 | 34.4 |
| L83V variant | 50 | 52.1 |
| Multivariant | 13 | 13.5 |
| Total | 96 | 100 |

Note: L83V variant was detected in higher frequency in all tested samples.

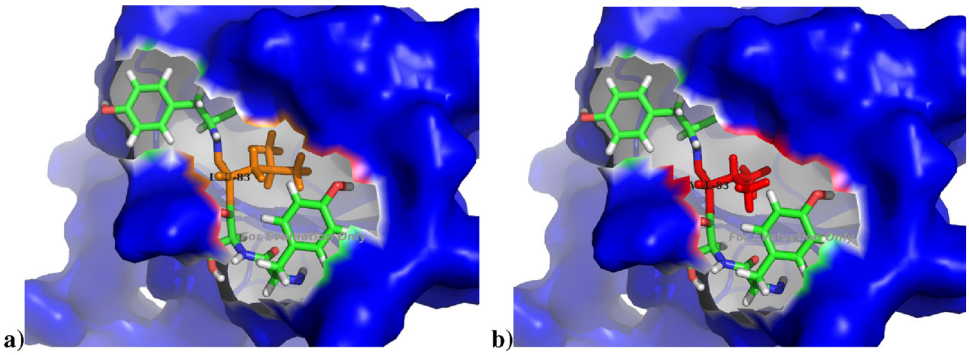


**Fig. 1 – 3D structure of HPV-16 E6 protein. (a) 3D structure represents the prototype E6 protein variant (in case of wt there is a leucine amino acid in the 83rd position); (b) 3D structure represents the most frequent mutative form of E6 protein – HPV-16 T350G genetic variant (leucine amino acid in the 83rd position due to T350G (or L83V) mutation is changed to valine). The diagram is generated by PYMOL software, using the solved three-dimensional HPV-16 E6 protein PDB data file ID:2LJX.**

However no statistically significant differences were found when compared HPV16 prototypes or L83V variants distribution separate in SCC or CIN3/Ca *in situ* cases ($P = 0.102$ and $P = 0.090$ respectively).

In the remaining cases, the prototype and L83V variant distributed as follows: 2 prototypes and 1 variant were detected in three cases of normal cervical samples (no cervical pathology confirmed), 2 prototypes in both CIN1, 1 prototype and 2 variants in three CIN2 cases and 1 prototype in single case of AD.

As we stated previously, 13 cases were described as multivariants. The multivariant defined as HPV 16 variant with 2 or more nucleotides changes in the sequence could not be attributed to any of the previously described prototype and L83V variant. Moreover, these nucleotide variations usually lead to the amino acid change. In our case, 9 samples of multivariants were associated with amino acid change, 4 others cases did not lead to the amino acid change. According to the histological diagnosis, 6 cases of multivariants were attributed to the SCC, 5 multivariants were found in CIN3/Ca *in situ*. One by one multivariants were found in CIN2 case and normal epithelium. It is worth noting that usually in cases of invasive cervical cancer more than 2 nucleotides changes were detected (4 and 5 changes in the individual cases); majority of these changes are leading to the amino acid change and, probably, to the transformation of viral oncogenicity. Fig. 2 shows all detected multivariants with polymorphic nucleotide sites and amino acid changes.

**Table 3 – HPV type 16 prototype and L83V variant distribution in CIN3/Ca *in situ* and SCC cases.**

| Histology | Prototype | % | L83V variant | % |
|---|---|---|---|---|
| CIN3/Ca *in situ* | 19 | 38.0 | 31 | 62.0 |
| SCC | 8 | 33.3 | 16 | 66.7 |
| Total | 27 | 36.5 | 47 | 63.5 |

CIN3/Ca *in situ*, cervical intraepithelial neoplasia grade 3 or carcinoma *in situ*; SCC, squamous cell carcinoma.
$P = 0.696$ when comparing difference of L83V variants between CIN3/Ca *in situ* and SCC; $P = 0.090$ when comparing the difference of prototype and L83V variants frequency in CIN3/Ca *in situ*; $P = 0.102$ when comparing the difference of prototype and L83V variants frequency in SCC.

### 3.2. *Analysis of HPV 16 E6 variants by the phylogenetic lines*

As discussed earlier, HPV 16 variants could be attributed to the various phylogenetic lines according their sequences and nucleotides changes: EUR, AFR-1, AFR-2, As, AA and NA. In our cases non-prototypic variants were attributed as follows: in 60 cases (50 cases of L83V and 10 cases of multivariants) were attributed to the European (EUR) phylogenetic line, 1 multivariant to the North American (NA) and 1 to the Asian-African (AA). Of all 13 multivariants, 1 could not be attributed to any of the known phylogenetic lines. Determination to the phylogenetic lines of our samples is presented in Fig. 3.

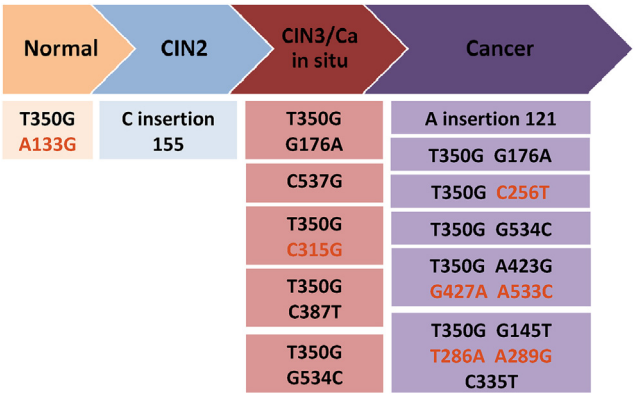


**Fig. 2 – Multivariants distribution according to histological diagnosis. Every block includes nucleotide substitutions which occurred in analyzed single case. Mutations which lead to amino acid changes are highlighted in black bold, while the red color denotes silent mutations which do not lead to the amino acid change in the sequence.**

| Phylogenetic lines | | HPV 16 E6 gene nucleotide positions | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 109 | 131 | 132 | 143 | 145 | 178 | 286 | 289 | 295 | 335 | 350 | 403 | 532 |
| | | T | A | G | C | G | T | T | A | T | C | T | A | A |
| European | EUR | -/C | -/G | - | - | - | -/A | - | - | - | -/T | -/G | - | - |
| Asian | AS | - | - | - | - | - | G/C | - | - | - | - | - | - | - |
| African | AFR1A | - | - | C | G | T | - | A | G | - | T | - | - | - |
| | AFR1B | - | G | - | G | T | - | A | G | G | T | G | - | - |
| | AFR2B | C | - | T | G | T | - | A | G | - | T | - | G | - |
| | AFR2A | - | - | - | G | T | - | A | G | - | T | - | - | - |
| North American | NA | - | - | - | - | T | - | A | G | - | T | G | - | - |
| Asian - American | AA1 | - | - | - | - | T | - | A | G | - | T | G | - | G |
| | AA2 | - | - | - | - | T | - | A | G | - | T | G | - | -/G |

**Fig. 3 – The combinations of HPV-16 genetic variant mutations and association with phylogenetic line. The table shows the main positions of E6 gene. Nucleotide mutations in these positions reveal the investigated genetic variant dependence to one of the phylogenetic lines −/X, where X = A, T, C or G represents the nt (nucleotides) changes that may occur or may not occur. Gray background denotes mutations which have the greatest diagnostic significance for phylogenetic assignment of the line. Different colors highlight the variants which were identified in this study: EUR variants (red color) *n* = 60; *n* = 1 of NA and *n* = 1 of AA2 (green color).**

## 4. Discussion

For many years cervical cancer incidence in Lithuania is one of the highest in Europe. The cervical cancer screening program was launched in Lithuania 2004. Ten years later the positive effect of the program was stated – the incidence of cervical cancer has stabilized and more cancer cases at early stages have been detected [13].

It is well known that HPV is one of the main cervical cancer risk factors. In many countries, the detection of the virus is included in screening programs. However, even using combination of cytological test with HPV detection in some cases it is difficult to predict progression of woman's disease. In these cases the new individual molecular markers could help to improve screening efficacy.

Today various molecular markers to improve cervical screening usually are associated with HPV identification and various modifications of this test. Women with cervical cancer are most commonly infected with HPV 16. Correlations between HPV prevalence, viral load and copy number of infected cells and disease progression were reported by various authors. Another test as viral integration status to the host genome could be used as a additional marker to predict disease outcome [1]. In addition to HPV type or integration status detection HPV 16 genetic polymorphisms are investigated in order to prevent developing of cervical changes [5,6]. Other researchers showed that identification of genetic variants and their classification according phylogenetic lines is very important in disease prediction [7–9]. The scope of the current study was to analyze the distribution of various HPV 16 variants in cases of cervical cancer and high-grade cervical intraepithelial neoplasia of Lithuanian women. Cases from previously conducted hospital-based HPV prevalence study were analyzed.

As described previously, HPV 16 variants are classified into two major groups based upon common phylogenetic patterns of SNP: European (EUR) and non-European (NE). Distribution of different variants is heterogeneous within the continents. In Europe the L83V variant is detected most frequently. However this distribution differs among European countries. In the Danish population [14], the EUR prototype was detected in 61.3% of the tested samples. The majority of Greek women were infected by the EUR variants (93%) and sequence of HM596520 was the most frequent (84.6%) [15]. Another study was performed in Slovenia [16]. Despite the most frequently detected L83V variant in Slovenia (60% of all cervical cancer cases) the authors reported that Slovenian women were carriers of some special HPV16 E6 T350G genomic variant carrying a 63-bp in-frame insertion in the E1 gene. The new study by French scientists [17] showed that majority of women with normal cytology were infected by the EUR prototype (40.8%) and L83V variant (51.4%). According to the authors HPV 16 prototype (EUR-350 T) was linked for persistence of the infection more often than L83V (EUR-350 G) variant (OR = 1.6, 95% CI = 0.8–3.4). The similar data were shown in the study from Denmark: infection with HPV 16 prototype (EUR-350 T)

was associated with a significantly increased risk for persistence (OR = 2.06, 95% CI = 1.04–4.25) [9]. Therefore, authors discuss about behavior of HPV16 variants and some important significance. However, many of them conclude that any clinical utility of variant analysis is not yet strongly evident.

In one of our previous studies [18] it was showed that European L83V variant was distributed in 63.0% of cervical cancer cases and in 44.0% in cervical cases with normal cytology. However, only few cases with normal cytology were analyzed and CIN cases were not included in this previous study. Today, comparing both our studies we could say that HPV 16 European variant L83V is still more prevalent in cervical cancer cases and, moreover, equally distributed in SCC and CIN3/Ca *in situ* cases. But numbers of investigated samples remain too small and data have no statistical significance. Despite a small number of investigated samples we could say that Lithuanian women usually are infected by EUR variants of HPV 16 and our data are similar with others studies conducted in Europe.

Another results we could find in the research from North and South America. In the previously conducted studies [19] authors stated that the distribution of HPV 16 (and HPV 18 as well) variants is closely associated with the racial groups. However EUR variants are more prevalent in all racial groups of American women (detected in 82% of all detected cases). Separately in white women EUR variants were detected in 86.5% of cases, in Africa Americans it was detected in 66.7% of cases; contrary AFR variants were detected in 4.3% and 26.5% respectively. Later the same group [20] described results from the ASCUS/LSIL Triage Study (ALTS), where 796 HPV16 positive women were included in the analysis. HPV16 EUR, As, NA, AA, AFR-1, and AFR-2 variants were detected in 82.1%, 0.8%, 0.8%, 7.7%, 4.5%, and 4.1% samples, respectively. A total of 291 women with HPV16 positive CIN3 cases from were included for the 2-year follow-up study. However, the authors conclude that lineages of HPV16 variants are associated with differing risks for high-grade CIN. In the new study by USA researchers [21] 86% of all cases were infected by the EUR HPV 16 variant with the distribution of prototype by 34% and L83V (350-G) variant by the 43% of cases. In this study, contrary, the authors conclude that EUR variants showed an increased association with severe cervical dysplasia or carcinoma.

Few studies were conducted in South America [22–24]. In the Argentinean population [23] in 68.2% of cases EUR and in 31.8% of cases AA variants were found. The non-prototypic EUR variant in these women was detected in 54.5% of cases. The authors discuss that the non-European (NE), but usually the Asian-American variants (AA), are more closely related to cervical cancer development. Regarding EUR branch, the non-prototypic variants also could be associated with the cancer development and disease progression. Phylogenetic analysis in the recent study from Brazil [24] identified distribution of 65.8% as HPV16 EUR and 34.2% as NE variants in analyzed 32 cases. The authors reported that NE types were associated with high-grade disease (CIN3+ *versus* <CIN3 OR = 4.6, 95% 1.07–20.2; $P = 0.05$).

Various data showed that the HPV 16 European variant is most widely prevalent worldwide except in Africa. However, it is worth noting that the EUR L83V variant is more frequently detected in some African populations. In the study by Qmichou et al. [25] the high prevalence of EUR variants in Moroccan women (58.3%) and especially with the most common detection of L83V (350 G) variant (65% of all EUR variants) was shown . On the other hand it could be mentioned that Morocco is geographically close to Europe. Maybe due to these reasons European virus types usually infect Moroccan women. In the Asia Pacific population, the EUR L83V variant was found with the high frequency and was associated with increased progression of cancer [26]. In the next study on Thai women the most prevalent variants were Asian (61% of HPV 16 positive cases). The European variant in this population was detected only in 7.3% of cases. New nucleotide variations of Asian variants showed 19–30-fold higher activity than the HPV 16 prototype [27].

Few studies were performed earlier in the Asian population. In the Chinese population 23.6% of HPV 16-positive cervical cancers samples belonged to the prototype, 65.5% were of the As variant, 5.5% were of AFR type 1 and 3.6% were EUR variants [28]. In North China from HPV 16-positive specimens, 67.31% belonged to the EUR lineage, while 32.69% were As variants [29]. These data again give us an assumption that EUR variants were transmitted to North China from Europe; therefore, the EUR variants in higher frequency were detected in North regions of China. The similar results were observed in Mongolia when the European prototype was detected in 66% of all cervical cancer samples [30]. The European prototype was also detected in 65.8% of cervical cancer samples from India [31]. The authors also mentioned about possible epidemiological linkage between Europe and India with regard to the transmission of HPV 16 infections to India.

Our study showed the similar results to other researchers. In our tested samples the EUR variants were detected most frequently. For women in our study the L83V variant was most common (52.1% of all HP V16 positive cases). Prototype (or 350 T variant) was detected in 34.4%; other variants were identified in the remaining 13.5% of all HPV 16 positive cases. Moreover, the L83V variant in similar and high frequency was detected in both cancer samples groups: it was documented in 66.7% of invasive cancer and 62.0% of CIN3/Ca *in situ* samples. We could make an assumption that the non-prototypic L83V variant could be associated with severe dysplasias (CIN3/CIS) and invasive cervical cancer as well. These results could help support the hypothesis and confirm findings of other authors [21] that the T350G mutation that causes change from leucine to valine in 83 position of HPV E6 gene leads to a higher oncogenicity of virus compared to the HPV 16 prototype. Also we agree that all EUR variants are associated with severe cervical dysplasia or carcinoma. On the other hand these results could be interpreted that mutations with amino acid changing lead to a more severe progression of the disease.

Our additional analysis of non-prototypic variants showed that 60 cases were attributed to the European phylogenetic line L83V variant with or without additional changes in the sequence (all attributed to the EUR line), and two cases were attributed to the NA (North American) and AA (Asian-American) phylogenetic lines (Fig. 3). On the other hand, NA and AA lines phylogenetically are very close; the position of nucleotide changes in the E6 gene in our cases is very similar. This example just proves the phylogenetic similarity of two

lines of HPV 16 in phylogenetic tree: NA and AA lines belong to the common phylogenetic branch [5]. Interestingly to note, that one case of multivariant in our study was not attributed to any of known today phylogenetic lines of HPV 16. It could be that additional sequencing of LCR region of this case could help assign this virus to exact phylogenetic line.

These our results confirm that the most prevalent HPV16 variants in the world belong to the EUR line. Since non-prototypic variants (in our case L83V) are usually associated with longer persistence of the infection, faster development of cervical lesions or cervical cancer progression, detection of viral mutations and different variants could have an important practical significance: it could be used as prognostic factors for cancer development or disease progression. Detection of not only HPV type but genetic variants together could help make decisions in the cervical cancer risk assessment or prevention the progression of existing cervical dysplasia to cancer. Identification of viral variants as additional markers could be included in the cervical screening programs or women's follow-up protocols.

Finally, these results could give us the more exact understanding about cervical cancerogenesis, population migration and virus transmission through continents or countries while these data could help better control HPV infections and incidence of HPV associated cancers.

## 5. Conclusions

HPV 16 variants belonging to the EUR phylogenetic line were detected in all our samples. In earlier Lithuanian population-based studies (cancer *versus* control) and this study (cancer *versus* CIN3/Ca *in situ*), a greater percentage of HPV 16 EUR L83V variant was found in women at high risk of cervical cancer, but statistically significant difference was not achieved when comparing difference of L83V variants between investigated groups and in the HPV 16 L83V variant and prototype distribution in CIN3/Ca *in situ* and cancer.

## Conflict of interest

All authors state that they have no any conflict of interest.

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