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MOLECULAR DIAGNOSTICS OF HUMAN PAPILLOMAVIRUS (HPV) AND STUDIES ON HPV PREVALENCE

Summary of doctoral dissertation

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VILNIAUS UNIVERSITETAS

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Introduction

Actuality of the problem. Human papilllomaviruses (HPVs) are common human pathogens that infect cutaneous or mucosal epithelia and may cause benign and cancer diseases. It is the most prevalent sexually transmitted virus in the world. This virus infects about 75% of sexually active women. HPV genotypes that are linked to the development of cervical neoplasia are classified as high-risk (HR) genotypes. Cancer of the cervix is the third most common cancer in women worldwide. The incidence rate of cervical cancer and mortality is one of the highest in Lithuania within Europe (Bray et al., 2002). Cervical cancer kills an average of 5 women every week in Lithuania.

There is no medical treatment for HPV infection, therefore detection of HPV genotypes is very important in predicting cervical cancer. For the laboratory diagnostics of premalignant lesions and cervical cancer the cytological test (Pap smear) is commonly used. However, the efficiency of the Pap smear test is limited with respect to sensitivity and specificity. During the last 20 years, modern PCR-based molecular diagnostics assays of HPV identification have been developed that guarantee high specificity and sensitivity of HPV detection. A significant geographic variation in the prevalence of HR HPV genotypes has been reported in different countries. The information about the prevalence of HR HPV in different geographic regions is essential for planning prevention measures by prophylactic HPV vaccines and for large-scaled screening programs based on HPV testing.

HPV is the most important factor in causing cervical cancer, but additional risk factors and genetic and epigenetic alterations are required for triggering the carcinogenesis. This emphasizes the importance to examine the relationship of the prevalence of HPV infection and potential risk factors of cervical cancer, such as the use of combined oral contraceptives, smoking and childbirth. Also, genetic and epigenetic changes in infected cells and the HPV itself may affect the development of cervical cancer and can be used as markers for carcinogenesis. The combination of these factors may lead either to the clearance of HPV infection or formation of benign or malignant lesions.

The aim of the dissertation was to develop and optimize molecular assays for HPV detection, to study the prevalence of HPV infection and HR HPV genotypes in selected clinical specimens and investigate the correlation between HPV infection and potential risk factors that may contribute to the development of cervical cancer.

Tasks of the dissertation:

1. To develop and improve PCR-based systems for the detection and genotyping of HPV.
2. To investigate the prevalence of HPV infection and HR HPV genotypes in the groups of Lithuanian women with known cytological and histological diagnosis.
3. To investigate the methylation pattern of selected DNA sequences of three HR HPV genotypes – HPV16, HPV18 and HPV51 and to evaluate the correlation between HPV DNA methylation pattern and the severity of cervical neoplasia.
4. To investigate the expression of cellular marker $p16^{INK4A}$ in cervical specimens and evaluate the correlation between $p16^{INK4A}$ mRNR expression level, HR HPV genotypes and the grade of cervical dysplasia.
5. To estimate the prevalence of HPV infection and HR HPV genotypes among cervical and oral epithelial samples of randomly selected healthy women.
6. To estimate the prevalence of HPV infection and HR HPV genotypes among oral epithelial samples of randomly selected healthy men.
7. To evaluate the contribution of HPV infection and possible risk factors such as the use of combined oral contraceptives, childbirth and smoking to the development of cervical cancer.

Scientific novelty

The research is interdisciplinary and involves development of molecular assays for HPV detection, HPV prevalence studies and investigation of potencial risk factors related to HPV infection.

We have developed and optimized the PCR assays based on previously described primer sets (GP5+/GP6+ and PGMY09/11) for the detection of general HPV infection and four multiplex PCR systems (MM-1, MM-2, MM-3, MM-4) for the detection of 16 HR HPV genotypes. In collaboration with "Thermo Fisher Scientific Baltics" we have designed new primers and developed a new PCR system (HR_HPV) for the identification of 16 HR HPV genotypes and assessed its efficiency by testing clinical samples.

We have performed the largest investigation on the prevalence of HPV infection and 16 HR HPV genotypes in the groups of Lithuanian women with known cytological and histological diagnosis. HPV16 was identified as the most common HPV genotype. Unlike to other countries, high prevalence of other HR HPV genotypes such as HPV31, HPV33 and HPV56 and low prevalence of HPV18 was demonstrated.

For the first time, we have investigated DNA methylation at 3′ parts of the L1 gene and the LCR sequence of HPV51 and compared to methylation pattern of HPV16 and HPV18. We demonstrated that the methylation of cytosines at CpG sites was more prevalent in the L1 gene than in the LCR sequence of all analyzed HPV genotypes and the frequency of HPV DNA methylation correlated with the severity of cervical neoplasia. The identified epigenetic alterations in HR HPV genomes may serve as a potential biomarker for HPV-induced carcinogenesis.

We have designed and optimized a new quantitative reverse-transcriptase PCR system for the detection of $p16^{INK4A}$ mRNA expression and evaluated the correlation between the $p16^{INK4A}$ mRNR expression level and the grade of cervical dysplasia. The increased $p16^{INK4A}$ mRNA expression in cervical samples with cytological and histological abnormalities can be used as a marker for an active HPV infection.

For the first time in Lithuania, we have investigated the prevalence of HPV infection and 16 HR HPV genotypes in oral epithelial samples of healthy men and women. We

demonstrated that the prevalence of oral HPV infection in men was more common than in women.

For the first time in Lithuania, we have investigated the correlation between the prevalence of HPV and factors possibly contributing to cervical cancer (the use of combined oral contraceptives, childbirth, smoking) and demonstrated that even the short-term use of contraceptives increases the incidence of HPV infection.

Defended propositions

1. The developed molecular methods for HPV detection and genotyping are suitable for investigation of clinical samples.
2. The prevalence of HPV infection and HPV16 genotype is correlating with the severity of cervical pathology.
3. The frequency of HPV16, HPV18 and HPV51 genomes methylation at 3′ part of L1 gene and LCR sequences is correlating with the severity of cervical neoplasia.
4. The levels of $p16^{INK4A}$ mRNA expression is correlating with the severity of cervical pathology.
5. The prevalence of oral HPV infection in men is more common than in women.
6. The use of combined oral contraceptive pills is a significant risk factor for HPV infectivity.

The contents of doctoral thesis. The doctoral thesis (in Lithuanian) contains the following parts: Introduction, Literature review, Material and Methods, Results and Discussion, List of references (219 citations), List of publications (6 positions), participation at conferences (7 positions), Figures (34), Tables (26). Total 130 pages.

Literature overview

Human papillomavirus (HPV) is a small, non-enveloped DNA virus that infects skin and mucosa and causes many benign and cancer diseases. The circular, double-stranded viral genome is approximately 8 kb in size and consists of early region encoding non-structural genes (E1-E7) responsible for virus replication, the late region encoding 2 late proteins (L1 and L2), which are the viral structural proteins and regulatory region (long control region, LCR). HPV contributes to the neoplastic progression of infected cells predominantly through the action of two viral oncoproteins, E6 and E7.

HPV infection cycle coincides with host epithelial cell differentiation. HPV infects the cells in the basal layer of the epithelium through damaged areas, then HPV DNA is transported into the nucleus. In this infection stage, virus is maintained in episomal state at a low copy number - about 50-100 copies per cell (Moody and Laimins, 2010). During differentiation of epithelial cells HPV infects the daughter cells. In the upper layers of the epithelium the viral genome is amplified to thousands copies per cell. The capsid proteins encapsidate the viral genome and virions are released with peeled karatinocytes (Pyeon et al, 2009). In this stage, HPV DNA can integrate into a host genome. The cell with integrated oncogenes E6 and E7 begins to produce these proteins by itself. E6 and E7 interact with various host regulatory proteins and influence the function or expression levels of host gene products, eventually leading to the disruption of the cell cycle. E6 and E7 respectively inhibit two key cellular negative regulatory proteins – p53 and retinoblastoma protein (pRb) (Saito and Kiyono, 2007).

More than 150 HPV genotypes are identified, 40 of which are known to infect anogenital tract (Bernard et al., 2010). HPV genotypes that are linked to the development of cervical neoplasias are classified as high-risk (HR) genotypes. The HR HPV genotypes have been detected in 99% of biopsy samples of cervical carcinoma that is the third most common cancer in women worldwide (Ferlay et al., 2008). HPV16 has been reported to be the most common HR HPV genotype worldwide. HPV18 is identified as the second most frequent HR HPV genotype (Bosch et al., 2008). Worldwide, the five most common HR HPV genotypes identified in HPV-positive women are HPV16, 18, 31, 58, and 52 (de

Sanjose et al., 2007). However, a significant geographic variation in the prevalence of HR HPV genotypes has been reported in different regions (Li et al., 2011).

There are three types of prophylactic HPV vaccines that protect against the 9 most prevalent HPV genotypes (6, 11, 16, 18, 31, 33, 45, 52, 58). Vaccination is the only way to protect against HPV infection, but it is effective if the person is not yet infected with the virus. Therefore, early diagnostics of HPV infection and identification of HR HPV genotypes is very important for prevention and treatment of cervical cancer.

Currently, the Pap smear is used as a standard test that identifies abnormal cervical cells. However, the efficiency of the Pap smear test is rather limited due to its low sensitivity and specificity. The Pap smear does not detect HPV infection (Murphy et al., 2003). Therefore, modern PCR-based assays are recently being introduced at clinical laboratories as complementary tests for molecular diagnostics of HPV infection and detection of HR HPV genotypes (Gravitt et al., 2000).

Materials and Methods

Clinical specimens and study groups. Cervical specimens were collected at the National Cancer Institute (Vilnius, Lithuania) from women (aged from 18 to 80 years) with known histological and/or cytological diagnosis. Other group of cervical specimens were collected from randomly selected women who visited the gynecologist for routine investigation (aged from 18 to 40) at the Lithuanian University of Health Science (LUHS). Oral epithelial samples were collected from randomly selected healthy women and men (aged from 18 to 40) at the LUHS. The studies were approved by the Vilnius Regional Committee of Biomedical Research (Lithuania, permission No. 158200-6-062-16) and Kaunas Regional Committee of Biomedical Research (Lithuania, permission No. B-2-14; 2013-02-11).

Oligonucleotides. Primers and probes were obtained from Metabion (Steinkirchen, Germany).

Cell lines. Cervical cell lines used in the study: CaSki, ATCC No. CRL-1550 (60–600 copies of HPV16 per genome) and HeLa, ATCC No. CCL-2 (10–50 copies of HPV18 per genome) and Chinese hamster ovary cell line (CHO, ATCC No. CCL-61) are stored at the Vilnius University Institute of Biotechnology, Department of Immunology and Cell Biology.

Plasmid pTZ57R and λ DNA. Recombinant plasmid pTZ57R and phage λ DNA samples containing amplicons of the HPV genotypes were obtained from „Thermo Fisher Scientific Baltics".

Specimen's collection and DNA purification. Cervical specimens were collected from the cervical epithelium using cervical brush. Oral epithelium specimens were taken with a swab from the mucosa of both cheeks. The brush heads were transferred into alcohol-based mediums: in-house prepared media (22% ethanol, 3% methanol, 18% isopropanol, 100 mM natrium acetate, 150 mM natrium chloride, 5 mM EDTA; 0.005% saponin, 0.2% zinc chloride) and in BD SurePath media, BD Bioscience. The swabs were transferred into alcohol-based media (Cymol, Copan). DNA was extracted from cervical and oral samples using "Sorpo clean^{TM} Genomic DNA Extraction Module" (Sorpo, Lithuania), "GeneJet^{TM}

Genomic DNA purification Kit" and "MagJET Genomic DNA Kit" (Thermo Fisher Scientific Baltics, Lithuania) following the manufacturer‘s protocols.

HPV DNA detection and genotyping. HPV DNA was amplified by the in-house optimized PCR using previously described general primer systems: GP5+/GP6+ and PGMY09/11 (Gravitt et al., 2000; Husman et al., 1995) and by our new in-house developed PCR system using a set of 19 primers specific for 16 HR HPV genotypes (HR_HPV PCR system) (Simanaviciene et al., 2015). The quality of isolated DNA was tested using specific primers of two house-keeping genes: the β-globin gene and prostate specific antigen (PSA) gene. As a positive control, the DNA extracted from HeLa cells was used. All HPV DNA-positive samples were genotyped by the in-house optimized multiplex PCR-based systems using four sets of specific primers for 16 HR HPV genotypes: 16, 18, 39, 58, 31, 33, 52, 56, 59, 68, 35, 45, 66, 51, 73 and 82 (Popendikyte et al., 2008). As a positive control for HPV genotyping, λ DNA samples containing HPV DNA of the respective HPV genotype were used. The amplification products were analyzed by electrophoresis in 2% agarose gels and visualized using ethidium bromide under UV transilluminator (ULTRA-LUM, USA).

Bisulfite DNA modification, PCR and DNA sequencing. DNA extracted from cervical specimens was used for bisulfite modification using "DNA Methylation-Gold Kit" (Zymo Research, USA) according to manufacturer's instructions. Three sets of primer pairs were used to amplify bisulfite-modified HPV16 (Kalantari et al., 2004) and HPV18 (Badal et al., 2004) DNA sequences containing 3′ part of the L1 gene and LCR. Four pairs of primers were designed to amplify HPV51 DNA sequences containing 3′ part of the L1 gene and LCR (Simanaviciene et al., 2015). The obtained PCR products were sequenced in DNA Sequencing Center of Institute of Biotechnology using "3730xl DNA Analyzer" (Applied Biosystems).

Extraction of RNA and DNA from clinical specimens and cDNA synthesis. The extraction of RNA and DNA from clinical specimens was performed as previously described (Chomczynski et al., 1987). The RNA extracted from the specimens was transcribed to copy DNA (cDNA) using "Maxima™ First Strand cDNA Synthesis Kit for RT-qPCR" (Thermo Fisher Scientific Baltics, Lithuania) according to the manufacturer's

protocols. The cDNA was used to determine the p16^{INK4A} expression level by quantitative PCR (qPCR). The DNA extracted from the same clinical specimens was used to determine HPV DNA and HPV genotypes.

Quantitative reverse transcription PCR (RT-qPCR). Recombinant plasmid pTZ57R with integrated p16^{INK4A} gene fragment was constructed and used as a template for preparing RNR transcripts, which were used to obtain standard curves for p16^{INK4A} mRNA quantitation. The cDNA prepared from clinical specimens was used to determine the p16^{INK4A} expression level in qPCR system. As an internal control, cDNA of integrin gene fragment synthesized using specific primers was used. The number of calculated copies of the p16^{ink4A} gene were divided by a number of calculated copies of the internal control (integrin gene) and multiplied by the coefficient (x1000).

Statistical analysis. All data were analyzed using Microsoft Excel 2010, SPSS 22.0 and Statistica 8 programs. To compare the efficiency of PCR systems, coeficient κ (kappa) was calculated. To estimate the association of cervical pathology with HPV infection and certain HPV genotypes, odds ratios (OR) and 95% confidence interval (CI) were calculated. Differences of HPV prevalence between the groups of specimens were compared using the χ^2 test. The frequency of methylation of CpG sites between different groups of the studied HPV DNA specimens were compared using Fisher‘s exact test. Expression of p16^{INK4A} mRNA between groups were compared using Kruskal-Wallis and Mann-Whitney tests. The p-value of 0.05 was considered statistically significant.

Results and Discussion

Optimization of PCR systems for HPV detection and genotyping

Genomes of HPV genotypes are very heterogeneous, so in order to identify as many genotypes as possible, optimization of PCR systems is very important. All specimens were analyzed for HPV DNA by two previously described conventional PCR systems: GP5+/GP6+ and PGMY09/11 (Gravitt et al., 2000; Husman et al., 1995). These PCR systems were newly optimized for a higher efficiency. In addition to these 2 PCR systems, in collaboration with "Thermo Fisher Scientific Baltics" we have designed new primers and developed a new conventional PCR system (HR_HPV) using a set of 19 primers specific for 16 HR HPV genotypes. The primers of HR_HPV PCR system were designed according to the bioinformatics analysis of HPV L1 gene sequences (Simanaviciene et al., 2015). Also, we newly optimized previously described multiplex PCR system that identify 16 HR HPV genotypes: 16, 18, 39, 58, 31, 33, 52, 56, 59, 68, 35, 45, 66, 51, 73 and 82. HPV genotyping was performed using four different special PCR master mixes (HPV-MM 1-4) and each of them consisted of four pairs of primers designed for different HR HPV genotypes (Popendikyte et al., 2008).

Sensitivity and specificity of PCR systems are very important, therefore optimal parameters were determined for all PCR tests. Firstly, optimal annealing temperatures, primers and *Taq* polymerase concentrations were determined. Then, effect of various additives (BSA, betaine, DMSO, glycerol) for PCR efficiency was determined. Finally, specific primers of house-keeping genes and optimal concentrations were determined. DNA extracted from HeLa cells (ATCC NO. CCL-2) was used for optimization of conventional PCR systems. Mixes of four phage λ DNA samples containing amplicons of the corresponding HR HPV genotypes were used for optimization of multiplex PCR system. Optimal parameters of conventional and multiplex PCR systems are shown in table 1 and 2, respectively.

Table 1. Optimal parameters of conventional (GP5+/GP6+/ PGMY09/11 and HR_HPV) PCR systems.

OPTIMAL PARAMETERS	PCR SYSTEMS		
	GP5+/GP6+	**PGMY09/11**	**HR_HPV**
Annealing temperature	50 °C	55 °C	60, 55, 50 °C
Concentration of primers	0.5 μM	0.15 μM	0.3 μM
Concentration of *Taq* polymerase	1 u	2 u	1.25 u
Additives	BSA 5 μg	BSA 1 μg	BSA 7.5 μg; betaine 0.3 M
Specific primers of house-keeping gene	β-gloF/R1 (β-globin gene)	PSA (IC 435+IC 1238) (prostate specific antigen gene)	β-gloF/R2 (β-globin gene)
Concentration of specific primers of house-keeping gene	0.3 μM	0.1 μM	0.1 μM

Table 2. Optimal parameters of multiplex (HPV-MM 1-4) PCR system.

OPTIMAL PARAMETERS	MULTIPLEX PCR SYSTEMS			
	MM-1	**MM-2**	**MM-3**	**MM-4**
Annealing temperature	72, 62 56 °C	72, 62 56 °C	72, 62 56 °C	72, 62 56 °C
Concentration of primers	0.3 μM	0.3 μM	0.2 μM	0.2 μM
Concentration of *Taq* polymerase	1 u	1 u	1 u	1 u
Additives	BSA 5 μg	BSA 5 μg	BSA 5 μg; betaine 0.5 M	BSA 5 μg

DNA extracted from HeLa cells was used to assess the sensitivity of conventional PCR systems. We found that sensitivity of GP5 +/GP6 + PCR system is 1 HeLa DNA copy (10-50 copies of the viral genome) and 5 HeLa DNA copies (50-250 copies of the viral genome) of PGMY09/11 and HR_HPV PCR systems.

Previously described conventional PCR systems (GP5+/GP6+ and PGMY09/11) were compared with the newly developed HR_HPV PCR system by testing a set of 108 cervical specimens collected from patients with cervical cancer. HR_HPV PCR system detected significantly higher frequency of HPV infection (91.6% HPV DNA-positive samples) as compared to GP5+/GP6+ and PGMY09/11 PCR systems (62.6% and 56.1% HPV DNA-positive samples, respectively) ($p<0.001$). The concordance between the PCR tests was poor ($\kappa \leq 0.2$), because different HPV genotypes are detected by PCR systems.

Therefore, HR_HPV PCR system developed and optimized in the current study are useful for molecular diagnostics of HR HPV infection.

The prevalence of HPV infection in the groups of women with known cytological and histological diagnosis

In this study, a total of 824 cervical specimens with confirmed histological and/or cytological diagnosis were analyzed for HPV DNA. All specimens were distributed into two groups: 547 specimens of women with various grades of cervical pathology (study group) and 277 specimens of healthy women (control group). All specimens were analyzed for HPV DNA by three PCR-based systems: PGMY09/11, GP5+/GP6+ and the newly developed HR_HPV PCR system (Simanaviciene et al., 2015).

In total, 437 specimens were positive for HPV DNA by either PCR test. As expected, the prevalence of HPV infection in women with cervical pathology was significantly higher than that in the control group (67.6% and 24.2%, respectively, $p<0.0001$).

To analyze the correlation between HPV infection and cytological alterations, all specimens with known cytological diagnosis were subdivided into four subgroups: 174 cases of atypical cells of undetermined significance (ASCUS), 67 cases of low-grade squamous intraepithelial lesion (LSIL), 244 cases of high-grade squamous intraepithelial lesion (HSIL) and 62 cases of squamous cell carcinoma (SCC). The prevalence of HPV correlated with the severity of cervical pathology ($p<0.0001$). In ASCUS, 50.0% of specimens were found to be HPV DNA-positive, in LSIL – 55.2%, in HSIL – 80.3% and in SCC – 80.6% (Fig. 1). The difference in HPV prevalence between the control group (healthy women) and all subgroups of specimens with cytological pathology ($p<0.0001$), the ASCUS and HSIL ($p<0.0001$), the ASCUS and SCC ($p<0.0001$), the LSIL and HSIL ($p<0.0001$), the LSIL and SCC ($p<0.001$) subgroups was statistically significant.

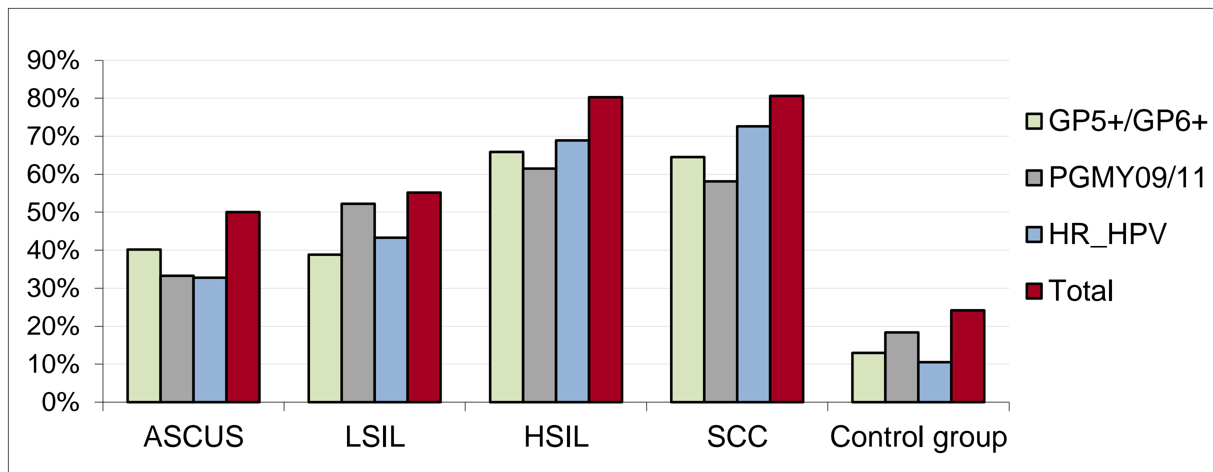

Fig. 1. The prevalence of HPV DNA-positive specimens among specimens with known cytological diagnosis analyzed by different PCR-based systems. "Total" indicates the prevalence of HPV DNA-positive specimens identified with all three PCR-based systems.

To analyze the correlation between HPV infection and histological alterations, the group of specimens with confirmed histological diagnosis was subdivided into five subgroups: 35 cases of cervical intraepithelial neoplasia grade 1 (CIN 1), 43 cases of cervical intraepithelial neoplasia grade 2 (CIN 2), 157 cases of cervical intraepithelial neoplasia grade 3 or carcinoma in situ (CIN 3/CIS), 75 cases of cervical cancer (Ca), and 133 cases of not confirmed pathology ("Normal histology"). The prevalence of HPV correlated with the severity of cervical pathology ($p<0.0001$). In the CIN 1 subgroup, 68.6% of specimens were found to be HPV DNA-positive, in CIN 2 – 86.0% in CIN 3/CIS – 89.2%, in Ca – 82.7% and in "Normal histology" – 41.4% (Fig. 2). Statistically significant differences were detected between all subgroups of specimens with histological pathology and both control (healthy women) and "Normal histology" subgroups ($p<0.01$), as well as between the CIN 1 and CIN 3/Cis subgroups ($p<0.01$). Similar rate of HPV DNA positivity in the advanced-stage disease is reported in other studies (Agoff et al., 2003, Smith et al., 2007).

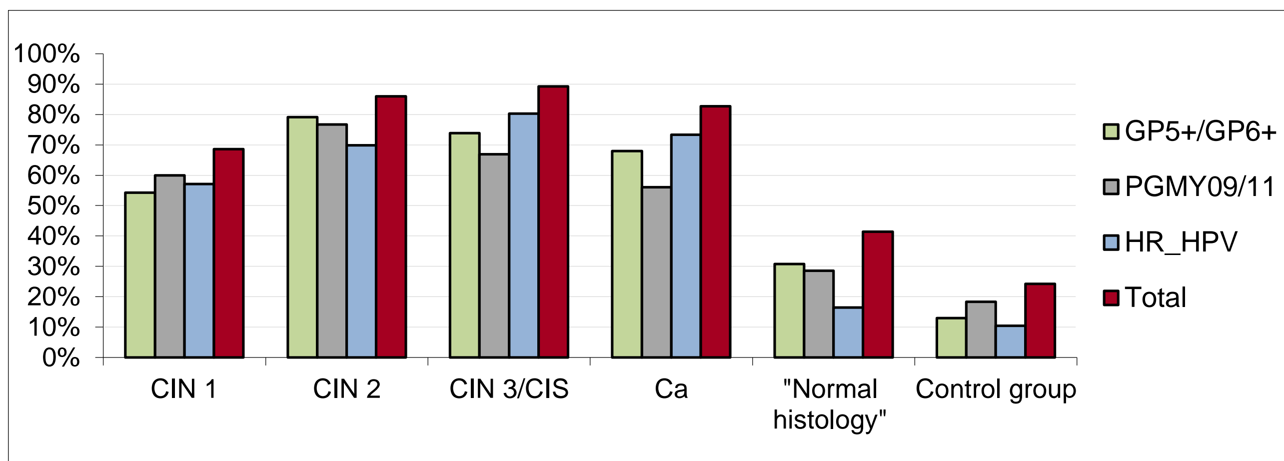

Fig. 2. The prevalence of HPV DNA-positive specimens among specimens with known histological diagnosis analyzed by different PCR-based systems. "Total" indicates the prevalence of HPV DNA-positive specimens identified with all three PCR-based systems.

Our study showed a relatively high prevalence of HPV DNA in cervical specimens collected both from the study and the control groups of Lithuanian women. The prevalence of HPV DNA in cervical cancer specimens was 82.7% in our study and was similar to that reported for other countries. According to the large meta-analyses, the HPV DNA-positivity rate was 79%–95% in cervical cancer, depending on the continent (Smith et al., 2007). The HPV-positivity rate in the group of healthy Lithuanian women was relatively high (24.2%) and similar to that reported in two previous studies: 26.7% (Gudleviciene et al., 2005) and 23.6% (Gudleviciene et al., 2006), respectively. Meanwhile, HPV incidence in other world regions is lower (Africa 22.1%, Central America and Mexico 20.4%, Northern America 11.3%, Europe 8.1% and Asia 8.0%) (de Sanjose et al., 2007).

The prevalence of HPV genotypes in the groups of women with known cytological and histological diagnosis

HPV DNA-positive specimens (n=437) were further analyzed for HPV genotypes using an in-house developed multiplex PCR system designed for the detection of 16 HPV genotypes (Popendikyte et al., 2008). Of the analyzed 437 HPV-positive specimens, 94 (21.5%) specimens were negative for any of the tested HPV genotypes. HPV16 was the most common HR HPV genotype, detected in 185 out of 437 (42.3%) HPV-positive

specimens (p<0.0001), followed by HPV31 (10.1%), 33 (8.2%) and 56 (5.7%). Only 3.9% of HPV-positive specimens were infected with HPV18 (Fig.3).

Our study revealed a relatively high number of HPV DNA-positive specimens with multiple HPV infection. We found that 94 specimens (21.5%) of all HPV DNA-positive specimens (n=437) had multiple HR HPV genotypes: more than one HPV genotype was detected in 82 (22.2%) specimens of women with abnormal cytology (study group) and 12 (17.9%) specimens with normal cytology (control group) (Fig. 3).

The prevalence of HPV genotypes was also analyzed in specimens subdivided according to the cytological and histological diagnosis. The frequency of HPV16 among HPV-positive specimens correlated with the severity of cervical pathology (p<0.0001). In the subgroup of cervical cancer HPV18 was the second most prevalent genotype after HPV16 (11.3% and 61.3%, respectively, p<0.05). Other HPV genotypes were distributed similarly among all subgroups divided according to the cytological and histological diagnosis (data not shown).

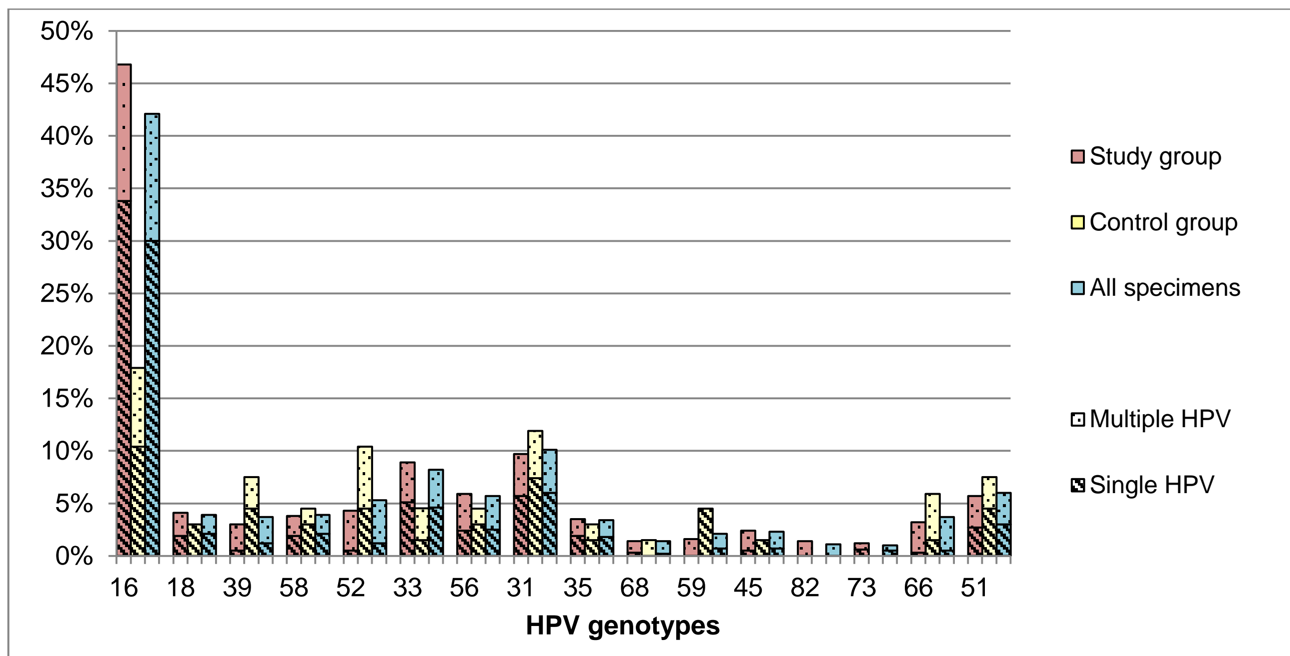

Fig. 3. The distribution of 16 analyzed HPV genotypes in a single (lined area) and in a multiple HPV infection among all HPV DNA-positive specimens. The study group represents women with cervical pathology and the control group represents healthy women with normal cytology.

According to the meta-analyses of HPV genotype distribution in cervical cancer, HPV16 is the most common, and HPV18 is the second most common genotype in all continents (Smith et al., 2007). Previous studies suggest that HPV16 and HPV18 are detected in approximately 70% of all cases of cervical cancer worldwide (Bosch et al., 2008). These data are in line with our study, indicating that HPV16 and HPV18 are the most prevalent HR HPV genotypes in the subgroup of women with confirmed cervical cancer (detected in 72.6% of HPV-positive cervical specimens). However, the frequency of HPV18 (3.9% of HPV-positive specimens or 11.3% of all studied cervical cancer specimens) was lower in our study as compared to other countries. According to the previous studies, HPV18 is detected in 17.2% of all cervical cancer specimens in Europe, 22.1% in North America and 15.3% in Asia (Pagliusi and Aguado, 2004). In all analyzed specimens, the frequency of other HR HPV genotypes (HPV31, 33 and 56) was higher than that of HPV18. Thus, our study demonstrated high prevalence of other HR HPV genotypes besides of HPV16 with a potential to cause precancerous pathology and cervical cancer in the groups of Lithuanian women. The obtained data confirm the geographic variability in the prevalence of HR HPV genotypes and provide useful information for planning prevention measures of cervical cancer.

The prevalence of HPV infection and HPV genotypes in samples of larynx cancer

The optimized conventional PCR systems (GP5+/GP6+, PGMY09/11, HR_HPV) were used to investigate the prevalence of HPV infection in oral epithelium specimens of larynx cancer patients (n=43). HPV infection has been detected in 20.9% of larynx cancer specimens. The obtained results are similar to that reported for other studies: HPV infection was diagnosed in 21.3% samples of larynx cancer in Europe, 13.8% in North America and 38.2% in Asia (Kreimer et al., 2005).

All specimens positive for HPV DNA were further analyzed for HR HPV genotypes by using a multiplex PCR system (Popendikyte et al., 2008). Of the analyzed 9 HPV-positive specimens, 6 specimens were negative for any of the tested HPV genotypes. In the remaining three samples three different HPV genotypes were detected (HPV31, 58, 39

infection together with HPV31). According to other studies, HPV16 is the most common genotype in samples of larynx cancer. HPV16 has not been determined in our study. However, HPV31, 58 and 39 genotypes are also found in other countries (Syrjanen, 2003).

Methylation pattern of HPV 16, HPV 18 and HPV 51 DNA target sequences

In the current study we investigated the methylation pattern of selected DNA sequences of three HR HPV genotypes (HPV16, HPV18, HPV51). DNA bisulfite modification and sequencing of target amplicons were used to determine the CpG methylation status of HPV16, HPV18 and HPV51 genomes at 3′ part of L1 gene and LCR sequences in DNA samples purified from cervical specimens with confirmed HPV infection (n=202). LCR region was divided into 3 parts: LCR 5′, transcriptional enhancer and E6 promoter. The degree of HPV DNA methylation was evaluated as a percentage of methylated CpG sites along the selected parts of HPV genomes (3‘part of the L1 gene and the LCR region) in the subgroups with identified cervical pathology. HPV DNA sequences were scored as methylated case if at least one CpG site of the region selected for investigation was methylated.

Methylation status of 17 CpG dinucleotides of the L1 3'and LCR HPV16 DNA sequences was investigated in 157 DNA samples isolated from HPV16-infected cervical specimens. The highest degree of HPV16 DNA methylation (35.5%) was determined along the 3‘part of the L1 gene in case of cervical cancer. Statistically significant differences in HPV16 DNA methylation of L1 region sequences were detected between the subgroups of asymptomatic HPV16 infection and cervical cancer ($p<0.05$). Similar pattern of HPV16 DNA methylation was detected within the promoter region sequences. The degree of DNA methylation ranged from 0% in HPV16 DNA samples representing CIN I to 32.3% in HPV16 DNA samples representing cervical cancer. DNA methylation levels at the other LCR regions (LCR 5' and enhancer) were low among all tested cervical HPV DNA samples (Table 3A).

Methylation status of 25 CpG dinucleotides of the L1 3'and LCR HPV18 DNA sequences was investigated in 21 DNA samples isolated from HPV 18-infected cervical

specimens. The current study revealed an extremely high methylation frequency of HPV18 DNA along the 3'part of L1 gene sequences. DNA methylation frequency reached 100% in the selected sequences of HPV DNA isolated from specimens with CIN 3/CIS and cervical cancer. In contrast, DNA methylation levels at the selected sequences of HPV18 LCR were low among all tested cervical HPV DNA samples (Table 3B).

For the first time, methylation status of 27 CpG dinucleotides of the L1 3'and LCR HPV51 DNA sequences was investigated in 24 DNA samples isolated from HPV 51-infected cervical specimens. This collection of HPV51-positive specimens included only 1 case of cervical cancer. In this single cervical specimen, all cytosines were methylated within the 3' part of L1 gene sequences (Table 3C).

Table 3. HPV16 (A), HPV18 (B) and HPV51 (C) DNA methylation frequency within the L1 3'and LCR regions.

A								
Group	L1 3'		LCR 5'		Enhancer		Promoter	
	no.	%	no.	%	no.	%	no.	%
CIN I (n=10)	0	0	0	0	1	10	0	0
CIN II (n=17)	1	5.9	2	11.8	3	17.6	2	11.8
CIN III/CIS (n=70)	17	24.3	10	14.3	16	22.9	18	25.7
Ca (n=31)	11	35.5*	8	25.8	7	22.6	10	32.3
"Normal histology" (n=29)	1	3.4*	1	3.4	2	6.9	1	3.4
B								
Group	L1 3'		LCR 5'		Enhancer		Promoter	
	no.	%	no.	%	no.	%	no.	%
CIN I (n=3)	1	33.3	0	0	0	0	0	0
CIN II (n=2)	0	0	0	0	0	0	0	0
CIN III/CIS (n=2)	2	100	1	50	0	0	0	0
Ca (n=6)	6	100	2	33.3	1	16.7	1	16.7
"Normal histology" (n=8)	1	12.5	0	0	0	0	0	0
C								
Group	L1 3'		LCR 5'		Enhancer		Promoter	
	no.	%	no.	%	no.	%	no.	%
CIN I (n=6)	0	0	0	0	0	0	0	0
CIN II (n=4)	0	0	0	0	0	0	0	0
CIN III/CIS (n=5)	0	0	0	0	0	0	0	0
Ca (n=1)	1	100	0	0	0	0	0	0
"Normal histology" (n=8)	0	0	0	0	0	0	0	0

*p<0.05, in accordance with Fisher's exact test.

The obtained data revealed that DNA methylation of 3‘part of HPV L1 gene is much more prevalent than that of viral LCR. Moreover, HPV DNA methylation frequency is much higher in DNA samples isolated from carcinoma specimens than in those isolated from cervical specimens with asymptomatic HPV infection. Previous studies on HPV16 DNA methylation reported similar positive correlation between the degree of methylation in the L1 gene and the severity of cervical pathology (Fernandez et al., 2009; Mirabello et al., 2012). The obtained data on the pattern of HPV18 DNA methylation are also similar to previous reports, demonstrating an intense (often exceeding 80%) methylation at CpG positions within the 3‘part of L1 gene sequences in DNA samples isolated from cervical cancer specimens, and very low methylation (often none), in the HPV18 LCR DNA sequences (Badal et al., 2004; Fernandez et al., 2009). For the first time, DNA methylation pattern of the selected parts of HPV51 genome was analyzed. This collection of HPV51-positive specimens included only 1 case of cervical cancer. Noteworthy, we detected methylated cytosines within the 3‘part of L1 gene sequences (from 6916 nt to 7021 nt) only in this single HPV51 DNA sample representing a case of cervical cancer (Table 3C). Methylation pattern of other HR HPV genotypes, such as HPV31, HPV33, HPV45, HPV52 and HPV58 have been analyzed in recent studies. Obtained data demonstrated that HPV DNA methylation frequency of the L1 gene is much more prevalent than that of the LCR sequences (Murakami et al., 2013). However, the biological basis for the association between HPV L1 gene methylation and cervical cancer progression is still unclear. A few studies showed that the L1 gene methylation is associated with HPV integration into cellular DNA (Kalantari et al., 2004; Bryant et al., 2014). HPV DNA methylation events can be interpreted as a host defense mechanism based on methylation of chromosomally integrated foreign DNA (retroviruses, transgenes, transfected adenovirus genomes) (Doerfler et al., 2001). The identified epigenetic alterations in HR HPV genomes may serve as a potential biomarker for HPV-induced carcinogenesis.

Studies on the expression of p16^{INK4A} mRNA in cervical dysplasias

In the curent study, we have investigated the expression of p16^{INK4A} at the mRNA level in cervical specimens by RT-qPCR system and evaluated the correlation between the p16^{INK4A} mRNR expression level, the grade of cervical dysplasia and HR HPV genotypes. In the first step, both DNA and RNA were isolated from clinical specimens. The isolated RNA was used to investigate the expression of p16^{INK4A} mRNA, whereas the isolated DNA was used to determine HPV infection and HPV genotypes.

In total, 567 cervical specimens from women with known histological and/or cytological diagnosis were included into the study. For studying the correlation between the p16^{INK4A} mRNA levels and cytological alterations, all specimens with known cytological diagnosis was divided into five subgroups: 62 cases of ASCUS, 21 cases of LSIL, 158 cases of HSIL, 44 cases of SCC and 63 cases of normal cytology (control group). As shown in Fig. 4, the highest p16^{INK4A} mRNA expression level was observed in HSIL (mean number of copies 6941) and the lowest mRNA expression level in LSIL and control group (mean 1624 and 1710, respectively). Thus, enhanced p16^{INK4A} mRNA expression was associated with high-degree cervical dysplasia. The difference in p16^{INK4A} mRNA expression between the HSIL and ASCUS, control group and the SCC and control group was statistically significant ($p<0.05$).

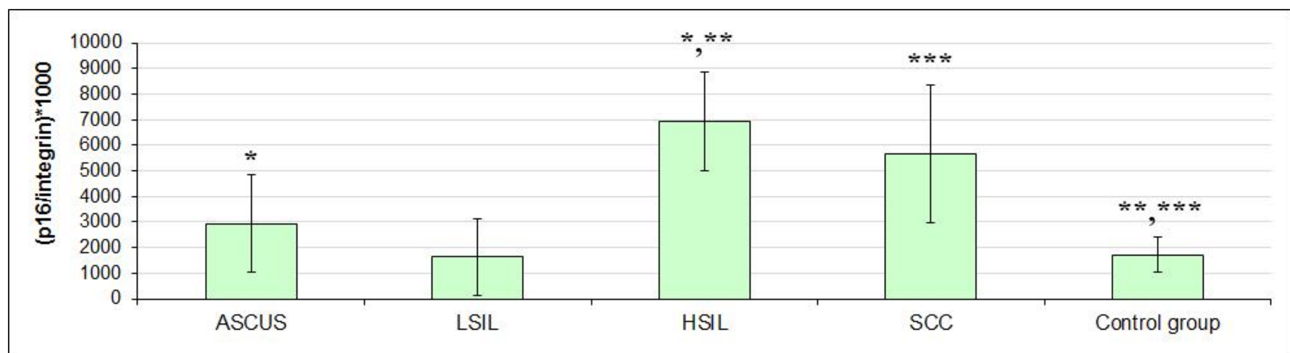

Fig. 4. Mean values and 95% CI of p16^{ink4A} mRNA copies in the groups of cervical specimens with confirmed cytological diagnosis.
*,**,*** $p<0.05$, in accordance with Kruskal-Wallis test (groups that were compared: * ASCUS and HSIL; ** HSIL and Control group; *** SCC and Control group).

For studying the correlation between the p16^{INK4A} mRNA levels and histological alterations, all specimens with known histological diagnosis were divided into five

subgroups: 15 cases of CIN1/CIN2, 111 cases of CIN3/CIS, 54 cases of cervical cancer (Ca), 21 cases with not confirmed pathology ("Normal histology") and 62 cases of normal cytology and histology (control group). As shown in Fig. 5, the highest level of $p16^{INK4A}$ mRNR was determined in CIN3/CIS specimens (mean 14065) and the lowest level in "Normal histology" and control groups (mean 449 and 1710, respectively). Surprisingly, the expression level of $p16^{INK4A}$ mRNA in specimens collected from patients with cervical cancer was lower than that in CIN3/CIS group (mean values 7067 and 14065, respectively). This suggests that the cellular processes leading to the up-regulation of $p16^{INK4A}$ as a tumour suppressor protein may be disrupted during the phase of tumour progression (Saito and Kiyono, 2007). The difference in $p16^{INK4A}$ mRNA expression between the two groups with histological alteration (CIN 3/CIS, Ca) and two groups without histological pathology ("Normal histology" and control group) was statistically significant ($p<0.05$).

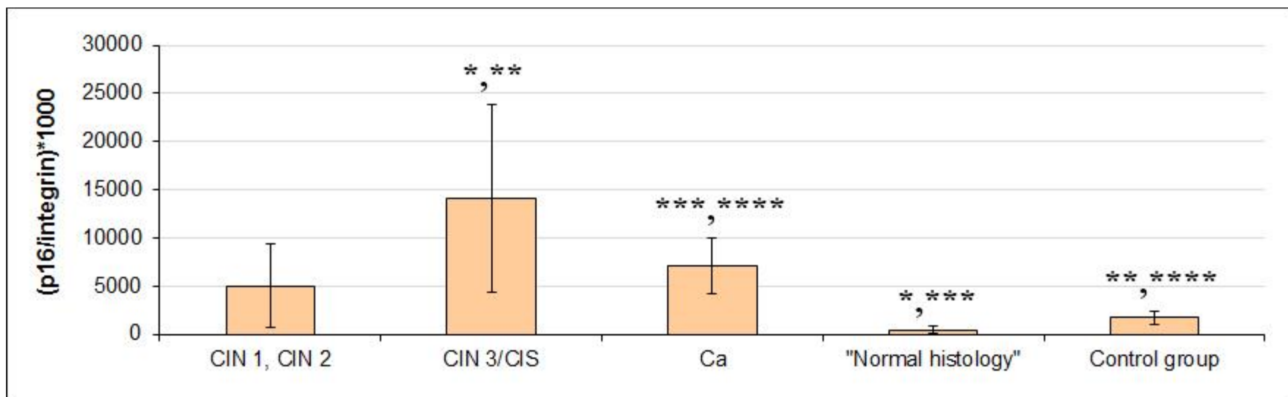

Fig. 5. Mean values and 95% CI of $p16^{INK4A}$ mRNA copies in the group of cervical specimens with confirmed histological diagnosis.
*,**,***,**** $p<0.05$, in accordance with Kruskal-Wallis test (groups that were compared: * CIN3/CIS and "Normal histology"; ** CIN3/CIS and Control group; *** Ca and "Normal histology"; **** Ca and Control group).

In summary, the correlation analysis of $p16^{INK4A}$ mRNA expression and cytological/histological alterations revealed a clear association between the enhanced $p16^{INK4A}$ mRNA levels and high-grade cervical dysplasias. These results are in line with previous studies that reported enhanced expression of $p16^{INK4A}$ protein in dysplastic squamous and glandular cells by immunocytochemical analysis (Dijkstra et al., 2010; Ishikawa et al., 2006). On the other hand, our study demonstrated that expression levels of

p16^{INK4A} mRNA may decrease during tumour progression thus leading to the possible false-negative results of the immunodetection.

As the disruption of the cell cycle leading to the oncogenic transformation of cervical cells is caused by HR HPV infection, we have investigated the correlation between the p16^{INK4A} mRNR expression levels and the particular HR HPV genotype. The specimens were analyzed for HPV DNA by two conventional PCR systems: PGMY09/11 and GP5+/GP6+ (Gravitt et al., 2000; Husman et al., 1995). In total, 275 (48.5%) specimens positive for HPV DNA were identified. They were further analyzed for HPV genotypes by using the multiplex PCR system (Popendikyte et al., 2008). 57 specimens out of 275 (21%) were positive for HPV16. More than one-half of the specimens (165/275) were negative for any of the tested HR HPV genotypes, which suggests that these specimens were infected with low-risk HPV genotypes. The correlation between the particular HR HPV genotype and expression level of p16^{INK4A} mRNA was analyzed (Fig. 6). However, no evidences on the correlation between a particular HR HPV genotype and expression level of p16^{INK4A} mRNA were obtained. This indicates that all HR HPV genotypes may cause pathology.

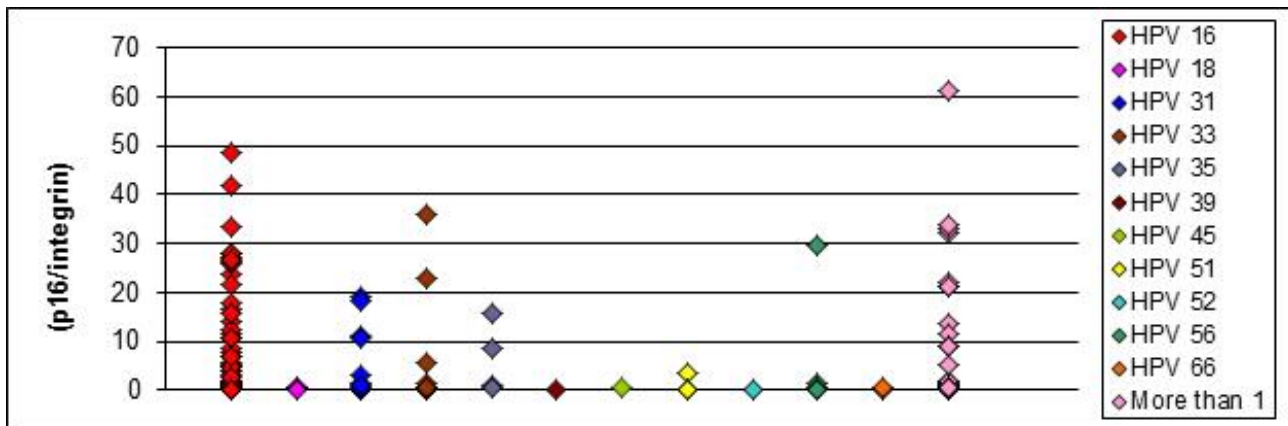

Fig. 6. Correlation analysis of HR HPV genotypes (x axis) and the levels of p16^{ink4A} mRNA expression (y axis).

The obtained results suggests that all HR HPV genotypes may dysregulate the cell cycle by a similar molecular mechanism. Therefore, it can be concluded that the oncogenic potential of HPV but not a particular HR HPV genotype is the main cause of enhanced

p16^{INK4A} mRNR expression indicating the dysregulation of cell cycle and leading to high-grade cervical lesions.

The prevalence of HPV infection and HPV genotypes in oral samples of healthy women and men

We have studied the prevalence of HPV infection and 16 HR HPV genotypes in cervical specimens of randomly selected women who visited gynecologist for a routine investigation. Similar study was performed with oral epithelial specimens of a group of healthy men and women. In Lithuania, detection of HPV infection and 16 HR HPV genotypes in oral specimens of healthy men and women was performed for the first time.

A total of 657 specimens were investigated for HPV DNA: 249 oral and 249 parallel cervical specimens of women (aged from 18 to 40 years) and 159 oral specimens of men (aged from 18 to 46 years). All specimens were analyzed for HPV DNA by three conventional PCR-based systems: PGMY09/11, GP5+/GP6+ and the newly developed HR_HPV PCR system. HPV DNA was detected in 47.8% of cervical specimens (Fig. 7). The estimated HPV prevalence among randomly selected women in Lithuania is higher than in other countries: in Denmark 33.4% of HPV-positive samples (Rebolj et al., 2014) and in Estonia – 36.8% of HPV-positive samples (Uuskula et al., 2010) were found.

The prevalence of oral HPV infection in a group of randomly selected men was 22.0%, while the prevalence of oral HPV infection in women was 9.2%. ($p<0.001$) (Fig. 7). Our study revealed that oral HPV infections are more common among healthy men than women, similar to other studies (Kreimer et al., 2005; Gillison et al., 2012). Most studies showed a broad variation in the prevalence of HPV infection among healthy men (ranging from 2.6% to 20.7%) (Kreimer et al., 2005). Higher HPV-positivity rate among men may suggest the influence of sexual behaviour to the prevalence of oral HPV infection.

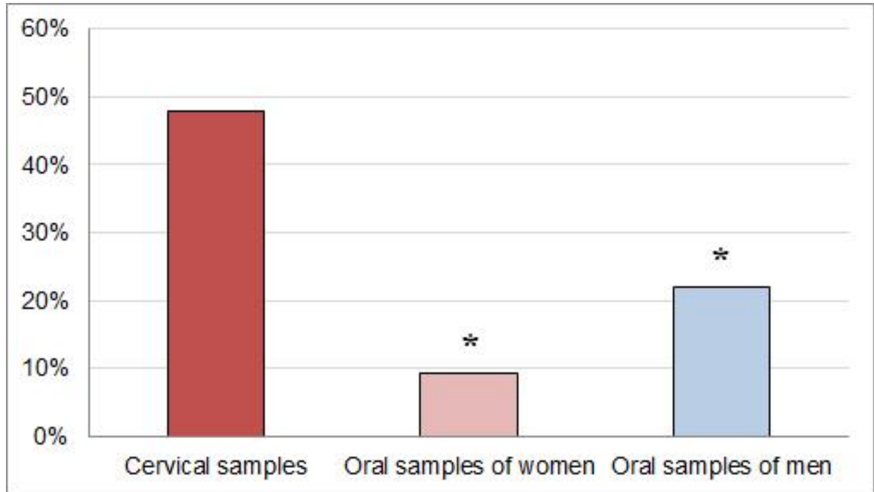

Fig. 7. The prevalence of HPV DNA-positive specimens among cervical and oral specimens of women and men.
*$p<0.05$, in accordance with χ^2 test.

A total of 177 specimens positive for HPV DNA were further analyzed for HR HPV genotypes by using a multiplex PCR system (Popendikyte et al., 2008). The most common HPV type in cervical specimens was HPV16 (detected in 17.7% of HPV-positive specimens) followed by HPV39 (9.2%), HPV31 (8.4%) and HPV66 (5.9%).

In oral specimens of women three different HPV genotypes in three samples (HPV 16, 66, 33) were detected. In oral specimens of men two HPV genotypes (HPV 66, 56) in four oral samples were identified (Fig. 8).

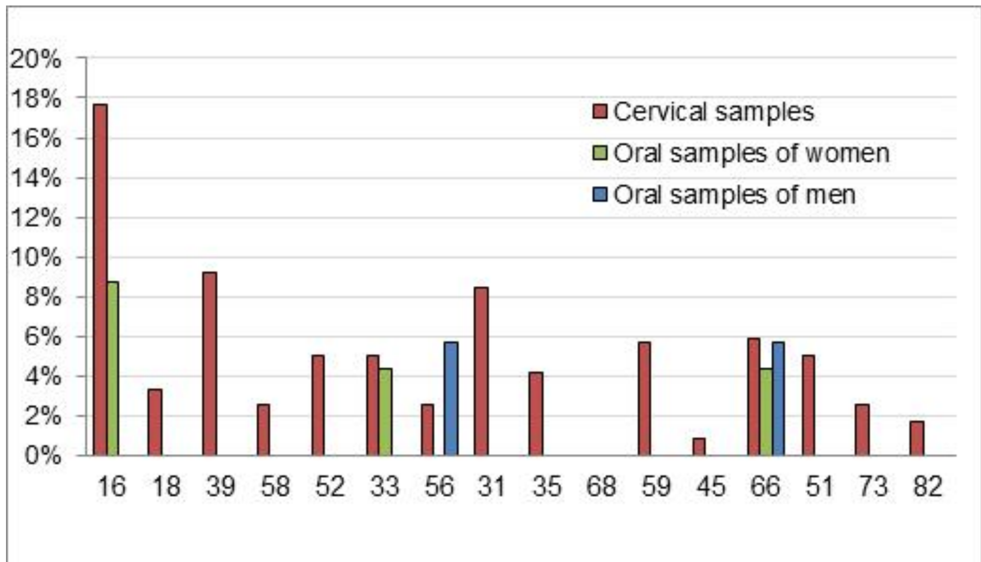

Fig. 8. The distribution of 16 analyzed HR HPV genotypes in cervical and oral samples of healthy women and oral samples of healthy men among all HPV DNA-positive specimens.

As demonstrated in our study, the prevalence of HPV16 (17.7%) in healthy women is suprisingly high, and comparable to the prevalence of HPV16 in other studies (19.1%) where only cases of cervical cytological abnormalities were analyzed (Bruni et al., 2015). HR HPV genotypes were identified only in a few oral samples of our study. This can be explained by the fact that HPV DNA is present in small amounts in oral swabs and therefore is not detectable by multiplex PCR systems that are less sensitive as compared to general HPV primer systems. Another explanation might be that oral samples are infected with low risk HPV genotypes.

Correlation between HPV infection and risk factors possibly contributing to cervical cancer

The aim of this study was to determine the corrrelation between HPV infection and possible risk factors of cervical cancer – the use of combined oral contraceptive pills (COCP), number of childbirths and smoking – in the same group of healthy women who were analyzed for HPV infection and HR HPV genotypes (n=249).

The first task of this study was to determine the correlation of the prevalence of HPV infection and HPV genotypes and the use of COCP. All cervical and oral samples of women (n=249) were divided into several groups: combined oral contraceptive pills users (COCP, n=67, duration of oral contraceptive use was 6 month), COCP non-taking (COCP control group, n=57) and women who visited gynecologist for a routine investigation but did not report COCP use ("random women", n=125).

Our study revealed a significantly higher HPV-positivity rate among cervical samples of COCP users than in the control group ($p<0.05$). HPV DNA was detected in 55.2% COCP users, in 31.6% COCP control group and in 51.2% "random women" group. The frequency of HPV infection in oral samples was similar between the subgroups (COCP – 10.4%, COCP control – 7.0%, "random women" – 9.6%) ($p>0.05$) (Fig. 9).

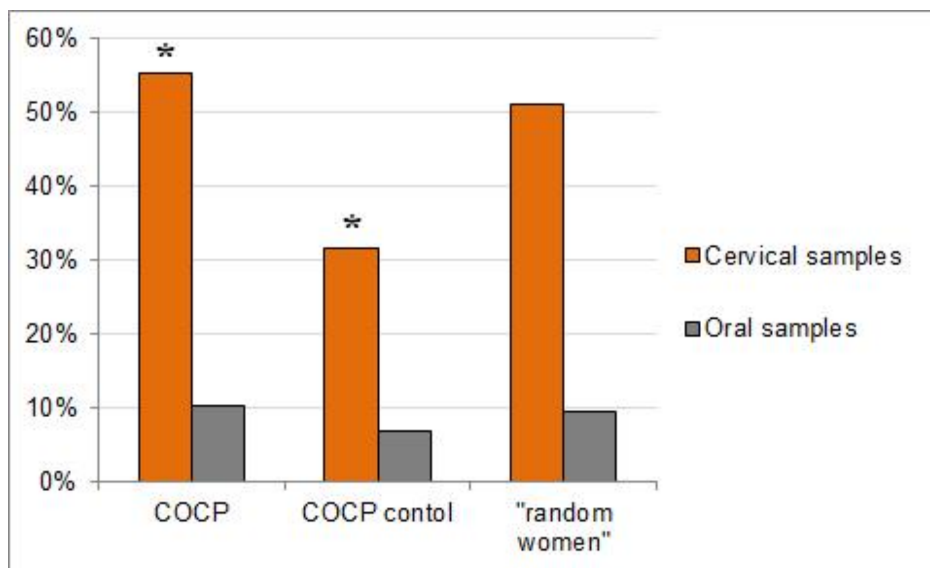

Fig. 9. The prevalence of HPV DNA-positive specimens among subgroups of combined oral contraceptive pills users (COCP), COCP non-taking (COCP control group) and randomly selected women who visited gynecologist for routine investigation (“random women”).
*$p<0.05$, in accordance with χ^2 test.

Other studies have shown the correlation between long-term use of COCP (>4 years) and higher HPV infection rate (Marks et al., 2011; Luhn et al., 2013). In our study, even the short-term (6 months) COCP use increases the incidence of HPV infection. It can be concluded that the use of COCP is a significant risk factor for HPV infectivity. However, it is unclear whether this association is due to COCP influence on HPV-dependent pathway of carcinogenesis or an acquisition of new HPV infection and increased duration of HPV persistence. The increased risk of HPV infection among COCP users may also suggest a possible influence of female sex hormones to HPV infectivity (Gariglio et al., 2009).

HPV DNA-positive cervical specimens (n=119) were further analyzed for HPV genotypes using the multiplex PCR system designed for the detection of 16 HR HPV genotypes (Popendikyte et al., 2008). Of the analyzed 119 HPV-positive specimens, 51 (42.9%) specimens were negative for any of the tested HPV genotypes. HPV16 was the most common HR HPV genotype, detected in 24.3% of HPV-positive specimens in COCP users, 11.1% in COCP control group and 15.6% in “random women” group (Fig. 10).

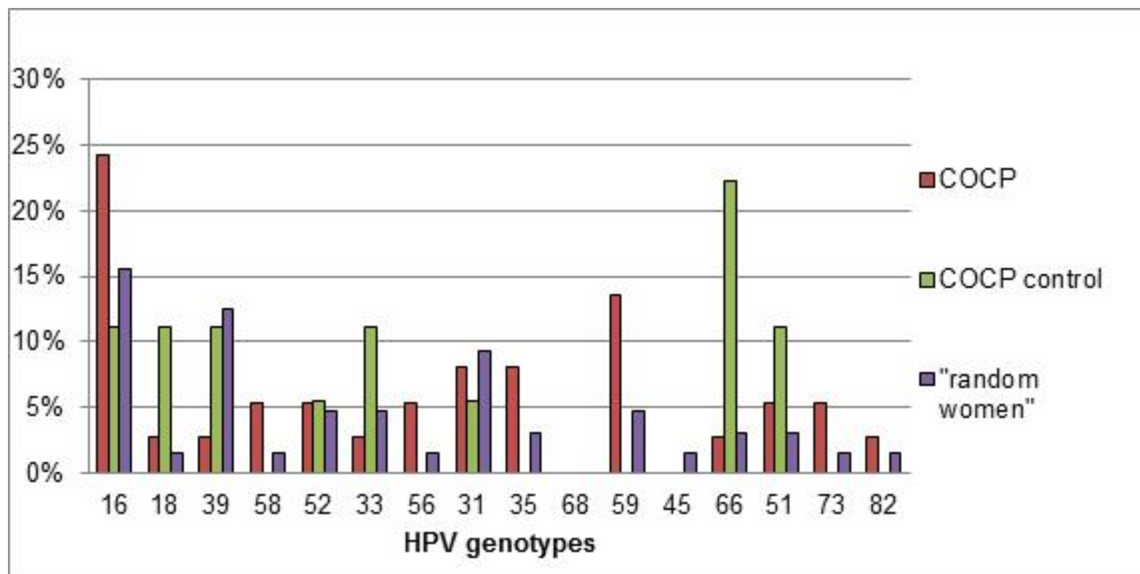

Fig. 10. The distribution of 16 analyzed HR HPV genotypes in cervical samples of combined oral contraceptive pills users (COCP), COCP non-taking (COCP control group) and random women among all HPV DNA-positive specimens ("random women").

Distribution of HPV genotypes between the groups is not statistically significant ($p>0.05$). The results indicate that HPV genotypes are distributed evenly between the groups, so it can be concluded that the use of COCP increases the risk of HPV infection, but does not increase the prevalence of a particular genotype.

The second task of this study was to determine the corrrelation of the prevalence of HPV infection and the number of childbirths. A total of 66 cervical specimens were investigated for HPV DNA and divided into two groups: 13 women who gave birth and 53 women who did not give birth. HPV infection was detected in 6 (46.2%) women who had delivery and in 31 (58.5%) women who did not have delivery ($p>0.05$) (Fig. 11). In order to evaluate the correlation between the number of births and the frequency of HPV infection, women were divided into two subgroups: women who had one delivery (n=10) and women who had two deliveries (n=3). In the subgroup of women who had one delivery was detected 3 HPV positive samples (30.0%) and in the group of women who had two deliveries, all three analyzed samples were HPV positive (100%). Due to the small number of samples, a statistically significant difference was not found ($p>0.05$).

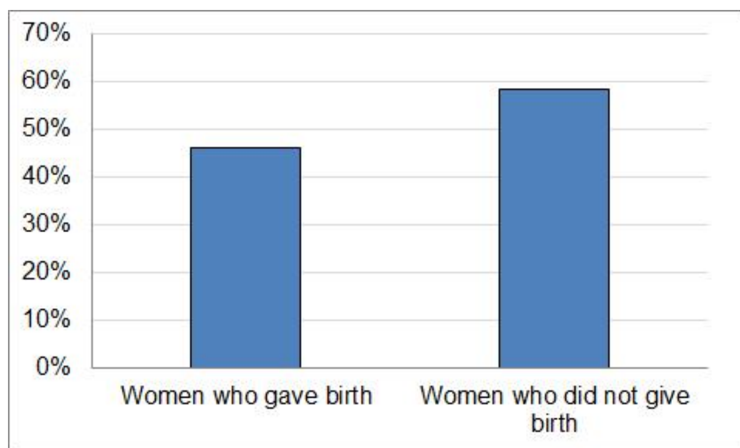

Fig. 11. The frequency of HPV DNA-positive specimens among groups of women who gave birth and did not give birth.

The literature data on the prevalence of HPV infection and number of childbirths are contradictory. One study showed a statistically significant difference between HPV prevalence and number of births (Castellsague et al., 2002; Jensen et al., 2013), others found no significant difference (Bardina et al., 2008). According to the literature, the risk of HPV infection increases 4-fold in women who have had 7 or more deliveries (Munoz et al., 2002). The increased risk might be due to higher hormone levels and reduced immunity during the pregnancy (Appleby et al., 2006).

The third task of this study was to determine the correlation of the prevalence of HPV infection and smoking. A total of 66 cervical specimens were investigated for HPV DNA and divided into two groups: 23 samples of smokers and 43 samples of non-smokers. HPV infection was detected in 15 samples of smokers (65.2%) and in 22 samples of non-smokers (51.2%) (Fig. 12). A statistically significant difference was not found ($p>0.05$). Comparing the frequency of HPV infection among women who smoke a different number of cigarettes, the samples were divided into two subgroups: women who smoke 1-6 cigarettes per day (n=12) and women who smoke more than 6 cigarettes per day (n=11). In the subgroup of women who smoke 1-6 cigarettes per day, 6 HPV positive samples were detected (50.0%) while in the subgroup of women who smoke more than 6 cigarettes 9 HPV positive samples (81.1%) were detected. Due to the small number of samples, a statistically significant difference was not found ($p>0.05$).

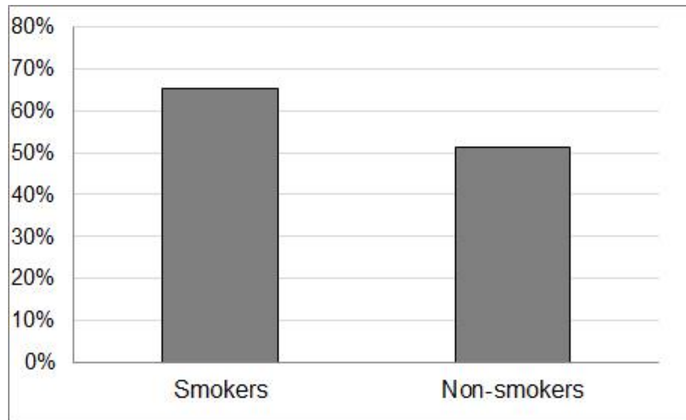

Fig. 12. The frequency of HPV DNA-positive specimens among groups of smokers and non-smokers.

Previous studies have reported controversial results on HPV infectivity and cigarette consumption. Some studies reported that HPV infection correlates with smoking rate (Castellsague et al., 2002; Sanders et al., 2012) and other studies declared no correlation between HPV infection and cigarette consumption (Ragin et al., 2011). It was found that the tobacco metabolites directly affect the cervical mucosa. These substances may inhibit the local immune response and contribute to a longer persistence of HPV in epithelial cells (Prokopczyk et al., 2009).

Conclusions

1. The new PCR system (HR_HPV) for the detection of high-risk HPV genotypes in a single sample was developed. Two previously described conventional PCR systems (GP5+/GP6+ and PGMY09/11) and the multiplex PCR system for the identification of 16 high-risk HPV genotypes were optimized. These PCR systems were proven useful for investigation of clinical specimens.
2. The prevalence of HPV infection and HPV genotypes was investigated in a large group of women with known cytological and histological diagnosis (n=824). Correlation of HPV infection and HPV genotypes with the severity of cervical pathology was demonstrated. HPV16 was the most common HPV genotype identified in 42.3% of HPV-positive specimens. High prevalence of HPV31 (10.1%), HPV33 (8.2%) and HPV56 (5.7%) was demonstrated. In contrast, the frequency of HPV18 (3.9%) was lower as compared to other countries.
3. DNA methylation pattern at 3′ parts of the L1 gene and the LCR sequence of HPV16, HPV18 and HPV51 was analyzed. For the first time, DNA methylation of HPV51 was investigated. The methylation of cytosines in CpG sites was more prevalent in the L1 gene than in the LCR sequence of all analyzed HPV genotypes. Moreover, the frequency of HPV DNA methylation correlated with the severity of cervical neoplasia.
4. The RT-qPCR assay for the analysis of $p16^{INK4A}$ expression was developed and the levels of $p16^{INK4A}$ mRNA were investigated in 567 cervical specimens. The levels of $p16^{INK4A}$ mRNA expression correlated with the severity of cervical pathology. However, no correlation between a particular HPV genotype and the type of pathology was demonstrated. This indicates that HPV oncogenic potential but not HPV genotype is associated with the increased levels of $p16^{INK4A}$ expression.
5. The prevalence of HPV infection and HPV genotypes was investigated in 249 cervical and oral epithelial specimens of randomly selected healthy women. About one-half of cervical samples (47.8%) were found to be HPV-positive. HPV16 was identified as the most common HPV genotype. The frequency of HPV infection among oral epithelial samples was 9.2%.

6. The prevalence of HPV infection and HPV genotypes was investigated in 159 oral epithelial samples of randomly selected healthy men. The prevalence of oral HPV infection in men (22.0%) was more common than in women (9.2%) ($p<0.001$).
7. The correlation between HPV infection and risk factors possibly contributing to cervical cancer was investigated. Higher HPV-positivity rate among cervical samples of women using combined oral contraceptives than in the group of non-users was determined (55.2% versus 31.6%, $p<0.05$). HPV16 was the most common HPV genotype in both groups. No statistically significant differences between HPV infectivity, childbirth and smoking were determined.

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1. **Simanaviciene V**, Popendikyte V, Gudleviciene Z, Zvirbliene A. Different DNA methylation pattern of HPV16, HPV18 and HPV51 genomes in asymptomatic HPV infection as compared to cervical neoplasia. Virology. 2015; 484:227-33.

2. **Simanaviciene V**, Gudleviciene Z, Popendikyte V, Dekaminaviciute D, Stumbryte A, Rubinaite V, Zvirbliene A. Studies on the prevalence of oncogenic HPV types among lithuanian woman with cervical pathology. J Med Virol. 2015; 87(3):461-71.

3. **Simanaviciene V**, Popendikyte V, Gudleviciene Z, Armalyte S, Kirkutyte A, Shikova E, Zvirbliene A. Studies on the expression of p16^{INK4A} mRNA in cervical dysplasias. J Biotechn Biotechn Equip. 2011; 80-84.

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Conference reports

1. 2016.06.28-30, „XIVth International Conference of Lithuanian Biochemical Society", Druskininkai, Lithuania. „Different DNA methylation pattern of HPV16, HPV18 and HPV51 genomes in asymptomatic HPV infection as compared to cervical neoplasia", (poster).

2. 2015.12.10, "LAS Conference of Young Scientist", Vilnius, Lithuania. „Studies on the prevalence of HPV and detection of epigenetic marker in clinical specimens", (oral presentation). ***3rd place and prize for the best presentation.***

3. 2015.06.14-17, ESHRE, Lisabon, Portugal. „A comparison of methylation levels in HPV16, HPV18 and HPV51 genomes in asymptomatic HPV infection and cervical neoplasia", (poster).

4. 2015.03.18-21, 16th World Congress on Human reproduction, Berlin, Germany. „Studies on the prevalence of oncogenic HPV types among Lithuanian women with cervical pathology", (poster).

5. 2014.06.17-20, „XIIIth International Conference of Lithuanian Biochemical Society", Symposium „Young biochemistry", Birstonas, Lithuania. "Studies on the prevalence of oncogenic HPV types among Lithuanian women with cervical pathology", (oral presentation).

6. 2014.06.11-13, „Global Virus Network Conference", Tallinn, Estonia. „Studies on the prevalence of oncogenic HPV types among Lithuanian women with cervical pathology", (oral presentation).

7. 2012.04.11, V National Doctoral Scientific Conference „Science – Health", Kaunas, Lithuania. „Molecular diagnostics of human papillomavirus (HPV) and studies on HPV prevalence", (oral presentation). ***1st place and prize for the best presentation in the section.***

Santrauka

ŽPV infekcija sukelia odos ir gleivinių pažeidimus bei vėžį. Gimdos kaklelio vėžio atveju iki 100 % biopsijos mėginių randama ŽPV infekcija. Lietuva pagal sergamumą gimdos kaklelio vėžiu ir mirtingumą nuo šios ligos pirmauja tarp ES šalių. Gydymo nuo ŽPV infekcijos nėra, todėl ŽPV infekcijos ir genotipų identifikavimas, vėžio rizikos veiksnių ryšio nustatymas bei vėžio žymenų tyrimai yra svarbūs prognozuojant vėžio išsivystymą bei kuriant vakcinas.

Šio darbo tikslas buvo ištirti ŽPV infekcijos ir genotipų paplitimą tarp Lietuvos moterų ir vyrų grupių ir nustatyti rizikos veiksnių bei karcinogenezės žymenų sąsajas su ŽPV infekcija.

1. **Darbo metu buvo** gauti šie nauji rezultatai: **1)** sukurta nauja PGR sistema (HR_HPV), skirta aukštos rizikos ŽPV genotipų nustatymui viename mėginyje. Optimizuotos dvi literatūroje aprašytas bendros ŽPV infekcijos PGR sistemos (GP5+/GP6+ ir PGMY09/11) ir dauginės PGR sistemos (MM-1, MM-2, MM-3, MM-4), skirtos 16 aukštos rizikos ŽPV genotipų identifikavimui; **2)** ŽPV infekcijos ir ŽPV genotipų dažnis ištirtas 824 moterų su žinoma gimdos kaklelio pakitimų citologine ir histologine diagnoze urogenitaliniuose mėginiuose. Nustatyta, kad ŽPV infekcijos dažnis koreliuoja su intraepitelinių gimdos kaklelio pakitimų laipsniu. Tarp aukštos rizikos ŽPV genotipų labiausiai paplitęs yra ŽPV 16 (identifikuotas 42,3 % ŽPV teigiamų mėginių), mažiau paplitę ŽPV 31 (10,1 %), 33 (8,2 %) ir 56 (5,7 %) genotipai. Skirtingai nei kitose šalyse, ŽPV 18 dažnis yra nedidelis – šis genotipas identifikuotas tik 3,9 % ŽPV teigiamų mėginių; **3)** ištirtas aukštos rizikos ŽPV 16, 18 ir 51 genotipų L1 3’ ir LCR DNR regionų metilinimo dažnis. ŽPV 51 DNR metilinimo tyrimas atliktas pirmą kartą. Visų trijų tirtų ŽPV genotipų DNR metilinimas dažniau nustatytas ŽPV L1 3’ geno sekoje nei LCR regione. Metilinimo dažnis koreliuoja su intraepitelinių gimdos kaklelio pakitimų laipsniu; **4)** paruoštas AT-kPGR metodas potencialaus vėžinio žymens p16^{INK4A} RNR raiškos tyrimui ir šiuo metodu ištirtas p16^{INK4A} RNR raiškos lygis 567 urogenitalinių nuograndų mėginiuose. Nustatyta, kad p16^{INK4A} RNR raiškos lygis koreliuoja su intraepitelinių gimdos kaklelio pakitimų laipsniu. Nerasta koreliacijos tarp konkretaus

ŽPV genotipo ir p16^{INK4A} RNR raiškos lygio, kas rodo, kad ŽPV onkogeninis potencialas, o ne konkretus ŽPV genotipas yra pagrindinis ląstelės ciklo reguliavimo pakitimus sukeliantis veiksnys; **5)** ištirtas bendros ŽPV infekcijos ir ŽPV genotipų dažnis 249 atsitiktinai surinktų sveikų moterų urogenitalinių nuograndų ir burnos epitelio mėginiuose. Urogenitalinių nuograndų mėginiuose nustatytas aukštas ŽPV infekcijos dažnis (47,8 % visų mėginių), tarp jų dažniausiai identifikuotas ŽPV 16 genotipas. ŽPV infekcijos dažnis moterų burnos epitelio mėginiuose sudarė 9,2 %; **6)** ištirtas ŽPV infekcijos ir genotipų dažnis 159 atsitiktinai surinktų sveikų vyrų burnos epitelio mėginiuose. Nustatytas ŽPV infekcijos dažnis vyrų burnos mėginiuose (22 %) yra aukštesnis lyginant su moterų burnos mėginiais (9,2 %) ($p < 0,001$); **7)** ištirtos sąsajos tarp įvairių rizikos veiksnių ir ŽPV infekcijos dažnio. Sudėtines kontraceptines tabletes vartojančių moterų grupėje nustatytas didesnis bendros ŽPV infekcijos dažnis (55,2 %), lyginant su nevartojančiųjų moterų grupe (31,6 %) ($p < 0,05$). Abiejose grupėse labiausiai paplitęs ŽPV 16 genotipas. Nenustatyta statistiškai reikšmingų sąsajų tarp ŽPV infekcijos ir rūkymo bei ŽPV infekcijos ir gimdymų skaičiaus.

Curriculum vitae

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