1 Background

Cervical cancer still remains the important cause of women morbidity and mortality. Almost 500,000 new cases are diagnosed every year worldwide, 270,000 of women died from this pathology. In the European Union (EU) 34,000 new CC cases and more than 16,000 deaths from this disease are reported annually. The highest annual world–standardized mortality rates are currently reported in the new EU member states as Romania and Lithuania (13.7/100.000 and 10.0/100.000, respectively), and the lowest mortality rate is reported in Finland (1.1/100.000) [1,2]. In the Lithuanian population cervical cancer still is in the leading position in cancer statistics and epidemiology. Every year approximately 500 new cases of cervical cancer are diagnosed in Lithuania. It is well–recognized that persistent infection of high–risk (oncogenic) human papillomavirus (HPV) is one of the most important risk factors for cervical cancer development. It is known that HPV16 plays the crucial role in cervical cancerogenesis. However, there are no clear established prognostic markers that would allow for predicting the progression of HPV16 infection to cervical cancer. For this reason, the determination of other risk factors and biomarkers besides of HPV infection could be important for risk assessment. One of the important risk factors for disease progression is the status of viral integration. The loss of certain HPV gene fragments indicate whether the virus is integrated into the host genome or if it is still in the episomal form. HPV integration into the cell genome causes the dysregulation of cell cycle, selective cell growth and faster proliferation. Viral integration is usually associated with the higher risk of the progression of cervical lesions to cancer. This conclusion is based on the observation that integrated virus forms are more frequently detected in cases of high grade cervical dysplasia or non invasive cervical cancer, such as carcinoma in situ or invasive cervical cancer [3–7]. The integration is characteristic for high–risk HPV types, especially HPV16 and HPV18. However, the data on the HPV integration status in cervical dysplasias are
controversial. Some of the previous studies indicate that complete virus integration is found in approximately 90% of cervical cancer cases. These studies report that the episomal forms of HPV16 are found exclusively in normal Pap smears or low-grade cervical dysplasia cases and they are not identified in cases of high-grade cervical dysplasia or cancer [5–7]. However, other studies indicate that integrated HPV forms are found also in cases of normal cytology. This suggests that HPV integration is an early event in cervical cancerogenesis [8–12]. Thus, previous studies provide controversial data on HPV16 integration status in normal cervical cells and cervical dysplasias or cancer. Different levels of HPV16 integration, different frequencies of integration in cervical cancer, precancerous lesions or normal cervical cells are reported [13, 14].

The aim of the present study was to evaluate the HPV16 integration status in two cases of cervical pathology: invasive cervical cancer (CC) and carcinoma in situ (CIS). For this purpose, we have investigated HPV16 E2 gene deletions in specimens with confirmed cervical cancer in comparison to carcinoma in situ. To evaluate the degree of HPV integration, three selected fragments of HPV16 E2 gene were amplified.

2 Materials and methods

2.1 Study population

Clinical specimens were collected in the period from September, 2010 to June, 2011. In total, 156 women attending the Institute of Oncology, Vilnius University (Vilnius, Lithuania) were included in the study. Sixty-six women were diagnosed with cervical cancer (CC) and 90 were diagnosed with cervical carcinoma in situ (CIS). Diagnoses of cervical cancer and CIS were confirmed by histology in the National Center of Pathology (Vilnius, Lithuania). The study was approved by the Vilnius Regional Committee of Biomedical Research (Lithuania, permission No. 158200–6–062–16). All women have signed the Patients Information and Agreements forms.

2.2 Collection of specimens and DNA extraction

All women included in the study were examined by gynecologist first: gynecological and cytological examination was performed for all women. Cervical samples were taken from the cervix using cervical brush. After cytological slide preparation remaining material was placed immediately in the transport buffer and transferred to the laboratory. The DNA was extracted from all samples using GeneJet™ Genomic DNA Purification Kit (Thermo Scientific Fermentas Ltd., Vilnius, Lithuania). After DNA extraction all samples were tested for the beta-globin gene (internal control for the presence of DNA) and for HPV positivity. The specimens positive for HPV DNA were subjected to HPV typing. DNA isolated from HPV16 positive samples was amplified for the detection HPV16 E2 gene deletions indicative for HPV integration degree into the host genome.

2.3 HPV detection and typing

All 156 samples were positive for beta-globin gene indicating an efficient DNA isolation and amplification. HPV DNA detection was performed using two sets of general HPV primers: GP5+/GP6+ and PGMY09/11 specific for DNA sequences within the L1 open-reading frame [15,16]. As a positive control, the DNA extracted from HeLa cells was used. The PCR was performed in 50 µl of PCR mix. DreamTaq™ Green PCR Master Mix containing DreamTaq™ DNA polymerase, optimized DreamTaq™ Green buffer, MgCl2, and dNTPs was used (Thermo Scientific Fermentas Ltd).

HPV positive samples were tested for HPV genotypes. The in-house developed and optimized multiplex PCR-based systems were used with four sets of primers specific for 16 HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73 and 82 [17]. As a positive control for HPV genotyping the recombinant plasmids containing HPV DNA of the respective HPV type were used.

PCR products were analyzed under UV transilluminator after electrophoresis in 2% agarose gel stained with Atlas ClearSight DNA Stain (Bioatlas, Estonia). All results were documented by photoimaging and stored in the computer.

2.4 Testing of HPV16 E2 gene deletion status

For the detection of HPV16 E2 gene deletion, PCR was performed using primers specific for the selected HPV16 E2 gene fragments. Three E2 gene fragments (475 base pairs (bp), 477 bp, and 276 bp) were amplified (Table 1, Figure 1). To perform the PCR analysis, the DreamTaq™ Green PCR Master Mix (Thermo Scientific Fermentas Ltd.) was used according to the manufacturer’s protocol [18]. SiHa cells were used as positive and HPV16 plasmids – as negative control for the E2 deletion. PCR products were analyzed under UV transilluminator after electrophoresis in 2% agarose gel stained with Atlas ClearSight DNA Stain (Bioatlas, Estonia). All results were documented by photoimaging and stored in the computer.
HPV16 integration status was classified according the number of E2 gene fragments amplified:
- 0 degree of integration – all three fragments of HPV16 E2 gene were amplified;
- 1st degree of integration – no amplification of 1 fragment;
- 2nd degree of integration – no amplification of 2 fragments;
- 3rd degree of integration – no amplification of all 3 fragments;

### 2.5 Statistical analysis

Statistical analysis was performed using SAS software. Comparison of categorical variables was performed using chi-squared test. The p-value of 0.05 was considered statistically significant.

### 3 Results

All women, included in the study, were sub-divided in different groups according to their age: till 25 years, 26–35 years, 36–45 years, 46–55 years, and over 56 years. In the CC group the majority of women (40.9%, n=27) were more than 56 years old. In the CC group, 24.2% of patients (n=16) were 46–55 years old, 22.7% (n=15) were 36–45 years old and 12.1% (n=8) were 26–35 years old. Average age in this group was 52.82±14.55years (±SD). In the group of patients with CIS diagnosis 4.4% (n=4) of patients were in the age group till 25 years, 34.4% (n=31) were 26–35 years old, 33.3% (n=30) were 36–45 years old, 17.8% (n=16) were 46–55 years old and 10% (n=9) were in the age group over 56 years old. Average age in the CIS group was 39.76±9.83 years (±SD). The majority of CC cases were diagnosed in women above 56 years (40.9%). In contrast, CIS was diagnosed more frequently in younger women: in the age groups of 26–35 years (34.4%, p=0.4091) and 36–45 years (33.3%, p=0.3317).

From 66 tested CC specimens 74.2% [95% PI: 63.64÷84.76] of specimens (n=49) were found to be HPV DNA positive. In CIS group 85.6% [95% PI: 85.53÷92.85] of specimens (n=77) were HPV DNA positive (p=0.0766). After HPV typing the most prevalent HPV type was found to be HPV16 in both groups of patients. In CC group HPV16 was confirmed for 48.5% of patients [95% PI: 36.43÷60.54] (n=32), in CIS group – for 50.0% of patients [95% PI: 39.67÷60.33] (n=45), p=0.8517. HPV18 was detected in 10.2% of patients [95% PI: 1.73÷18.67] (n=5) in CC group and only in 2.6% [95% PI: 0.95÷6.15] (n=2) of patients with CIS diagnosis (p=0.0692). In CIS group the other most frequent HPV types were HPV33 (10.4%, n=8), HPV31 (9.1%, n=7), HPV58 (6.5%, n=5), in CC cases – HPV39, 45, 56, 59 (4.1%, n=2 of each type). Other HPV types detected in our study are shown in Figure 2.

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**Table 1:** Primer sequences for the amplification of HPV16 E2 gene fragments.

| HPV16 E2 gene fragment | Primer sequences | Nucleotide (nt) position | Amplification product |
|------------------------|------------------|-------------------------|----------------------|
| Amplimer A 1 fragment  | A1 5’-AGGACGAGGACAAGGAA-3’ | nt 2735–2753 | 475 bp |
| Amplimer B 2 fragment  | B1 5’-TTTGGAAGAACATCGACTA-3’ | nt 3172–3193 | 477 bp |
| Amplimer C 3 fragment  | C1, 5’-GTAATAGTAAACCTACACCCAATA-3’ | nt 3597–3618 | 276 bp |

**Figure 1:** Agarose gel electrophoresis demonstrating the amplification of HPV16 E2 gene fragments. Notes: 475 bp, 477 bp, 276 bp – HPV16 E2 gene fragments; BT-483, 484, 485, 486, 490 – samples numbers; BT-485 deletion – no amplification of 276 pb fragments.
All HPV16 DNA–positive samples were subjected to PCR analysis for the detection of HPV integration status. For the evaluation of HPV16 integration, three HPV16 E2 gene fragments were amplified: 475 bp, 477 bp, and 276 bp. In both CC and CIS study groups in the majority of specimens all three HPV16 E2 gene fragments were amplified that indicates the presence of the episomal HPV form (0 degree of integration). The episomal HPV16 form was found in 71.9% [95% PI: 56.33÷87.47, p=0.0005] (n=23) of specimens in CC group and 75.6% [95% PI: 63.05÷88.15, p<0.0001] (n=34) of specimens in CIS group. Deletion of one HPV E2 gene fragment (1st degree of integration) was determined in 12.5% (n=4) of CC specimens and in 13.3% (n=6) of CIS specimens. Deletion of two HPV E2 gene fragments (2nd degree of integration) was determined in 6.3% (n=2) and 8.9% (n=4) of CC and CIS specimens, respectively. The CC and CIS groups differed according to the 3rd degree of integration (deletion of all three E2 fragments): 3rd degree of integration was observed in 9.4% [95% PI: 0.71÷19.51, p=0.0001] of CC specimens (n=3) and only in 2.2% [95% PI: 4.28÷6.64, p<0.0001] of CIS specimens (n=1) (Table 2). However, the difference between the studied two groups (CC vs CIS) was not statistically significant (p=0.1634) due to the small number of specimens with detected 3rd degree of HPV16 integration.

4 Discussion

Persistent infection of oncogenic HPV types is one of the well–recognized risk factors for cervical neoplasia and cancer development. Many studies show high HPV prevalence (close to 100%) in cervical cancer patients and the lower prevalence in cervical neoplasia cases [19–21]. In this study, 74.2% of specimens collected from CC patients and 85.6% of specimens from CIS patients were positive for HPV DNA. Interestingly, double HPV testing of the specimens with two different primer sets (GP5+/GP6+ and PGMY09/11) revealed lower prevalence of HPV DNA in CC cases as compared to CIS cases (p=0.0766). In contrast, our previous studies showed the highest prevalence of HPV in CC patients – 92.7% [22]. The prevalence of HPV DNA in the CIS study group was similar to that observed previously – 84.8% [23]. Few of other studies also reported the highest prevalence of HPV in CC patients and lower rates of HPV positivity in patients with cervical intraepithelial neoplasia in accordance to the grade of dysplasia [21]. One of the reasons of the lower HPV positivity in CC specimens could be virus absence in the late stage of cervical cancer (probably due to the necrosis) or insufficient number of cancer cells infected by HPV in tested specimens. Likewise HPV infection was

![Figure 2: The prevalence of different HPV types in cervical cancer (CC, n=32) and cervical carcinoma in situ (CIS, n=45) study groups. Note: CC - cervical cancer, CIS – carcinoma in situ.](image-url)
not detected in older women with CC. This fact also could state the natural clearance of HPV in older patients after cervical cancer was developed. On the other hand, HPV testing was performed from the remaining cervical cells after cytological slide preparation. It could influence insufficient number of cells infected by HPV. We also cannot exclude the presence in the specimens of HPV subtypes that are not detectable by the GP5+/GP6+ and PGMY09/11 PCR systems.

In both CC and CIS groups the most prevalent HPV type was HPV16 that was detected in 48.5% and 50.0% of CC and CIS patients, respectively. Similar results were obtained in many other studies across the world. The prevalence of HPV16 is approximately 50.0% of all HPV positive CC or cervical neoplasia cases. Therefore, it is supposed that HPV16 plays the crucial role in cervical cancerogenesis [21, 24–27]. Other important risk factors for cell cycle dysregulation is the status of viral, especially HPV16 or HPV18, integration into the host genome. Previous studies that employed the amplification of the HPV16 E2 gene from cervical lesions demonstrated the failure of amplification of one or more fragments, indicating E2 gene disruption. The E2 gene deletions were significantly more frequent in invasive squamous cell carcinomas than in CIN III lesions [18]. In our study, in the majority of CC and CIS cases the amplification of all three HPV16 E2 gene fragments was observed that indicates the presence of the episomal virus form. The episomal HPV16 form was found in 71.9% and 75.6% of tested CC and CIS specimens, respectively. However, the difference between the CC and CIS groups was not statistically significant probably due to the low number of specimens analyzed.

Our previous study was pointed on the integration status in the cases of all grades of precancerous lesions [23]. According to these results, in all cases of the confirmed cervical pathology (from CIN I to CIN III) the predominance of the mixed virus integration forms was found that is characterized by the deletion of one or two HPV16 E2 gene fragments. In the same study the failure of amplification of all three HPV16 E2 fragments was detected in 40% of cases, when cytological changes were not confirmed by histology [23]. This study is pointed exclusively on cancer. Thus, the results of our previous and the current investigations confirmed the viral integration in normal cervical epithelium or in CIN I cases thus suggesting that HPV16 integration is an early event in the cervical cancerogenesis. Other studies indicate the presence of both – integrated and episomal – HPV forms in cervical cancer [28–31].

Similar results on HPV16 integration were obtained by using different methodological approaches such as quantitative PCR assay (qRT–PCR) [14]. Analysis of the viral integration status using the qRT–PCR showed that completely integrated forms of HPV16 were detectable as a relatively rare event in CIS (8.7%) and CC (15.2%) cases. In contrast, the episomal and mixed HPV forms were commonly detected. Other authors [28, 32] have suggested that the detection of HPV16 integration status through evaluating the HPV–16 E6/E2 ratio by qRT–PCR is a highly sensitive method that might be useful in assessing the risk of cervical cancer. On the other hand, it was demonstrated that the E2/E6 qRT–PCR assay was not suitable to detect the full range of HPV16 integration events due to the common presence of HPV16 episomal forms in the majority of CC specimens [14].

| Diagnosis | Integration status | CIS n | % | CC n | % |
|-----------|-------------------|------|---|------|---|
| 0 degree of integration | (amplification of all three HPV E2 gene fragments) | 34 | 75.6 | 23 | 71.9 |
| 1st degree of integration | (no amplification of one HPV E2 gene fragment) | 6 | 13.3 | 4 | 12.5 |
| 2nd degree of integration | (no amplification of two HPV E2 gene fragments) | 4 | 8.9 | 2 | 6.3 |
| 3rd degree of integration | (no amplification of all three HPV E2 gene fragments) | 1 | 2.2 | 3 | 9.4 |
The age of the patients is also considered as the risk factor for the progression of cervical neoplasia. In previous studies, the integration of HPV16 was only rarely demonstrated in young women [33]. Furthermore, HPV integration was not found to be associated with virus persistence. These results also need to assess the rate of integration and to clarify the role of HPV integration on its persistence at early stages of infection [33]. We have not analyzed the results on HPV16 integration status according to women age due to insufficient number of cases in different age groups. All cited authors conclude that more longitudinal studies are needed to clarify the role of the onset of HPV integration and its correlation with disease progression. On the other hand, new risk factors and prognostic markers could be included in the studies for a better prediction of the outcome of HPV infection. Factors such as the status of telomerase-related genes, TERT and TERC and others could be investigated to assess the risk of the progression of cervical intraepithelial neoplasia [34, 35].

In the current study, we have investigated a relatively small number of clinical specimens (n=156) collected in Lithuania as the number of CC cases in the country is not high (approximately 500 per year). Therefore, the data obtained in the current study could suggest the limited diagnostic value of HPV16 integration status for the risk assessment and prediction of the cervical neoplasia progression. On the other hand, these data could be used for the future meta-analysis of the potential risk factors in HPV–induced cancerogenesis. In the different studies various methods of integration status detection were used, different materials were investigated. One of the authors analyzed cervical cells, others – cervical material from biopsy or conization. This discrepancy in the results could be associated with these factors. So, it is clear that it is the demand to stratify and uniform the different methods to the implementation this test to the clinic.

Finally, our results indicate the presence of both – episomal and integrated – HPV16 forms in cervical cancer and in carcinoma in situ. Integration status could not be used as additional test to distinguish between invasive and non invasive cervical cancer. There is reason to investigate the impact of integration in the further studies and evaluate some prognostic value for patients follow-up. On the other hand these results will help scientist to increase their knowledge in the field of HPV related cancerogenesis.

## 5 Conclusions

The current study revealed HPV DNA positivity in 74.2% and 85.6% of specimens collected from patients with diagnosed CC and CIS, respectively. The most prevalent HPV type in both study groups was HPV16 that was found in 48.5% and 50.0% of CC and CIS specimens, respectively. The episomal HPV16 form was detected in the majority of CC and CIS specimens. The deletion of all three HPV16 E2 gene fragments was detected in 9.4% of CC specimens and 2.2% of CIS specimens.

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