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The role of mRNA E6/E7 HPV high oncogenic risk expression in colposcopy of cervical intraepithelial neoplasia (CIN)

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Additional key words:
mRNA HPV
colposcopy
cervical intraepithelial neoplasia - CIN

Dodatkowe słowa kluczowe:
mRNA HPV

kolposkopia
śródnabłonkowa neoplazja szyjki macicy - CIN

The aim of this paper is the evaluation of colposcopy and mRNA E6/E7 HPV detection - as the marker of persistent human papilloma virus (HPV) infection in the triage of abnormal Pap smears and in the assessment of cervical intraepithelial neoplasia progression risk. The clinical material consisted of 85 women, participating the national cervical cancer screening in the period of April 2010, and October 2010, reffered to the Outpatient Clinic of Gynecologic Oncology and Female Genital Tract Neoplasms Prophylaxy of the Jagiellonian University Medical College in Krakow, Poland. All subjects were offered gynecological evaluation, Pap smear, colposcopy, DNA HPV (Hybrid Capture2, Qiagen) and mRNA E6/E7 testing (NulciSens, Biomerieux). In case of positive tests colposcopically directed cervical biopsy with histopathologic evaluation were performed. Results: The presence of mRNA E6/E7 HPV transcripts correlated with high grade squamous intraepithelial lesions, statistically significantly. There was statistically difference between colposcopic, histologic concordance comparing to mRNA E6/E7 HPV colposcopic histologic concordance ($p<0.001$). Conclusions. The presence of mRNA E6/E7 HR HPV may be assumed as specific marker of high grade cervical lesions. The combination of mRNA E6/E7 HR HPV ewith colposcopic evaluation increases the colposcopy concordanece with final histologic findings.

Cel pracy: Celem pracy jest ocena celowości połączenia kolposkopii oraz detekcji mRNA E6/E7 HPV (human papillomavirus)-uznanego markera przetrwałej infekcji HPV o wysokim potencjale onkogennym w weryfikacji nieprawidłowych wyników cytologicznych, oraz w ocenie ryzyka progresji śródnabłonkowej neoplazji szyjki macicy. Materiał i metody: badaniem objęto 85 pacjentek w okresie od kwietnia 2010 do października 2010 skierowanych do Poradni Ginekologii Onkologicznej i Profilaktyki Nowotworów Narządu Płciowego UJCM w Krakowie w ramach etapu pogłębionego programu profilaktyki raka szyjki macicy. U wszystkich Pacjentek wykonano badanie ginekologiczne, cytologię, kolposkopię,HPV DNA techniką HC2 (hybryd capture 2), ocenę E6/E7 mRNA HR HPV (NulciSens, Biomerieux). W razie stwierdzonych nieprawidłowych wyników wykonywano biopsję pod kontrolą kolposkopii z następową oceną histopatologiczną. Wyniki: Obecność mRNA E6/E7 HR HPV w znamiennym statystycznie stopniu korelowała z obecnością zmian dużego stopnia. Wykazano zamienną statystycznie różnicę w zgodności wyników badania kolposkopowego z ostatecznym wynikiem badania histopatologicznego w porównaniu do kombinacji badania kolposkopowego z detekcją mRNA E6/E7 HR HPV ($p<0,001$). Wnioski: Obecność mRNA E6/E7 HR HPV można uznać za wysoce specyficzny marker zmian dużego stopnia w obrębie szyjki macicy, jego kombinacja z badaniem kolposkopowym zwiększa zgodność tego badania z ostatecznym wynikiem badania kolposkopowego.

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Introduction

Squamous cell carcinoma (SCC) of the cervix is the second most common cancer in women and the leading cause of cancer-related death in females from underdeveloped countries. Each year, approximately 500,000 cases of cervical cancer are diagnosed worldwide.

Routine screening has decreased the incidence of invasive cervical cancer in the United States, where approximately 13,000 cases of invasive cervical cancer and 50,000 cases of cervical carcinoma in situ (i.e. localized cancer) are diagnosed annually. Cervical SCC arises from the metaplastic epithelium of the transformation zone (TZ) (squamocolumnar junction) and develops slowly through progressive dysplastic changes to carcinoma in situ (CIS) and invasive cancer. Cervical intraepithelial neoplasia (CIN) is divided into three stages according to the degree of epithelial dysplasia and differentiation. Lesions are accessible to colposcopic evaluation and biopsy, which makes monitoring disease progression relatively easy. Low grade lesions (i.e., CIN1 and, in some cases, CIN2) may spontaneously regress or not progress further, while the malignant potential of CIN 3 is 36% over 20 years [23].

There is considerable controversy regarding the possible over-treatment of patients with mild cervical abnormalities, with lesions being often excised or ablated. Thusly, identifying the markers of potentially malignant lesions would be of a great prognostic value [25]. Adenocarcinoma accounts for approximately 20% of invasive cervical cancers and its incidence is increasing notably among women aged less than 35 years, with no evidence of a reduction in the number of diagnoses since the introduction of mass screening programs [36,40,41].

The most common types of HPV found in cancer patients are types 16, 18, 31, 33, and 45 [3]. Persistent infection with these types is regarded as the earliest carcinogenesis stage [26]. The role of HPVs in the etiology of cervical cancer is tightly correlated with the overexpression of two oncogenes (E6 and E7) due to a specific opening in the E2 open reading frame in the integrated viral genome [18]. Studies of cervical cancer cell lines and cancer biopsy specimens have shown that the continuous expression of these genes is a necessary condition for the transformation and maintenance of neoplastic and dysplastic cells [35,43,44].

In recent years, many studies have shown that testing for HPV DNA can improve the detection of high-grade squamous intraepithelial lesions (HSILs) and SCCs [19]. This suggests that DNA testing can make a useful contribution to the triage of women with an equivocal cytology finding and for follow-up after the treatment of precursor lesions [32]. However, the high prevalence of transient and asymptomatic HPV infections means that DNA tests have low specificities. Identification of the persistent infections likely to produce high-grade lesions currently requires repeated monitoring of the HPV DNA types. Commercial nucleic acid sequence-based amplification in a real-time format allows the reliable type-specific detection of E6 and E7 mRNA from HPV types 16, 18, 31, 33, and 45. Several authors have thus suggested that RNA-based assays could be more effective than DNA testing in risk assessment [11,13,14, 22,23,24,38]. In the current study, we test this hypothesis using colposcopic, cytological and histological findings.

Table I
Cytological findings for the 85 women enrolled in the study.

Cytology results	No. (%) of women	Age (yr)
Unsatisfactory	0	0
Normal	12 (14.1%)	23-81 (42.8)
ASCUS	26 (30.5%)	22-59 (38.7)
LSIL	27 (31.7%)	21-73 (36.7)
HSIL	18 (21.1%)	19-60 (34.3)
SCC	0	
AGC	2 (2.35%)	35-56 (45.4)
Total	85 (100%)	-

Materials and Methods

Study subjects and collection of specimens. Specimens were collected from April 2010 to October 2010 from patients admitted for secondary screening to the Colposcopy Outpatient Service and the Gynecological Oncology Unit (Jagiellonian University Medical College, Krakow, Poland). The study group consisted of 85 women between 19 and 81 years of age (median age, 37,8 years;). 45.8% percent were between 30 and 39 years of age, 24.7% were under 30 years of age, and 29.4% were over 40 years of age. The age at first intercourse lay in the range of 15 to 22 years (median, 18.1 years), with 57% of the patients having their first intercourse between 15 and 18 years of age. The number of partners was ≥ 5 for 16.8% of the women, and 75,9% of the women were nonsmokers. The Ethics Committee of Jagiellonian University Medical College approved the study protocol. Written informed consent was obtained from all participants. All participants received a self-administered questionnaire requesting personal data, a gynecologic history, and information on exposure to risk factors. Pregnant women and women undergoing treatment for invasive cervical cancer were excluded. All patients underwent cytology, colposcopy, and sampling for subsequent testing for HPV. In cases in which colposcopy suggested the presence of suspicious lesions, biopsy specimens were taken. Cytology was based on a conventional Pap smear. The cytological diagnosis was made by specialized cytopathologists using the Bethesda classification system. Colposcopy was performed by specialized gynecologists. The results were reported following guidelines issued by PSCCP (The Polish Society of Colposcopy and Cervical Pathophysiology) member of EFC (European Federation of Colposcopy) and IFCPC (International Federation of Cervical Pathology and Colposcopy). Histology was performed with specimens collected by colposcopy-directed biopsy (traditional punch biopsy specimens) and/or cone specimens collected by the loop excision procedure. Histology results were obtained for all patients. Cervical specimens for nucleic acid analyses were collected with a cervical brush by standard procedures. The material was preserved in PreservCyt/ThinPrep solution (Cytyc Corporation, Boxborough, MA). Analyses were performed by the Virology Laboratory at the University Hospital, Krakow, Poland.

Nucleic acid isolation. Each ThinPrep sample was divided into two aliquots (4 ml and 10 ml), which were used for DNA and RNA detection, respectively. The aliquot used for analysis of HPV mRNA was centrifuged. Total nucleic acid was extracted from the concentrated cell pellet by the off-board protocol with the NucliSens easyMAG platform (bioMe´rieux, Poland), according to the manufacturer's instructions. The nucleic acids were eluted in 55 _l of elution buffer. Aliquots were appropriately stored for further processing.

HPV DNA was genotyped for HPV types 16, 18, 31, 33, and 45 by multiplex PCR. Each assay used 5 pmol of eluted nucleic acids and 20 pmol of each of the four primer sets. The reaction mixture contained 25 pmol of HotStart Taq master mixture (Qiagen, Germany) and 10 pmol of RNase-free water in a final volume of 50 pmol. The amplification profile consisted of 15 min at 95°C to activate the HotStar Taq DNA polymerase (Qiagen), followed by 45 cycles of denaturation (94°C for 30 s), annealing (56°C for 40 s), and extension (72°C for 40 s). Assays were performed on an iCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Amplicons were detected by electrophoresis of 20 pmol of the amplification products in a 2% agarose gel and were visualized by ethidium bromide staining under a UV light transilluminator (Fluor-S). The molecular sizes of the amplicons (for HPV type 16 [HPV-16], 152 bp; for HPV-18, 216 bp; for HPV-31, 513 bp; for HPV-33, 455 bp; and for HPV-45, 124 bp) were determined by matching them against commercial DNA molecular size markers (molecular weight markers V and VIII; Roche Diagnostics, GmbH, Mannheim, Germany).

Table II
Colposcopic pictures, IFCPC classification.

Colposcopy not satisfactory	5 (5.8%)
Colpsocopy satisfactory, correct image	7 (8.23%)
Colposcopy satisfactory Images suggesting minor changes	43 (50.58%)
Colposcopy satisfactory Images suggesting major changes	30 (35.29%)

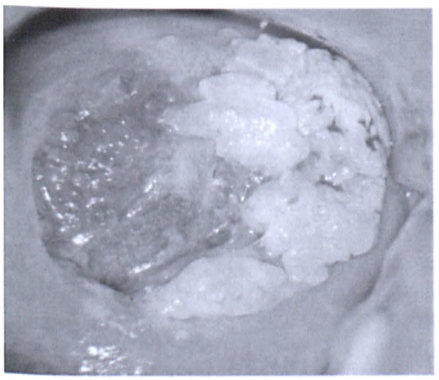
Table III
Histology results.

Histology results	No. (%) of women	Age (yr)
Normal - benign	5 (5.8%)	22-32 (28.3)
CIN1	44 (51.7%)	21-59 (36.3)
CIN2	20 (23.5%)	22-81 (41.9)
CIN3 (CIS)	14 (16.4%)	26-60 (36.5)
SCC	2 (2.3%)	19-78 (48.5)
Total	85 (100%)	-

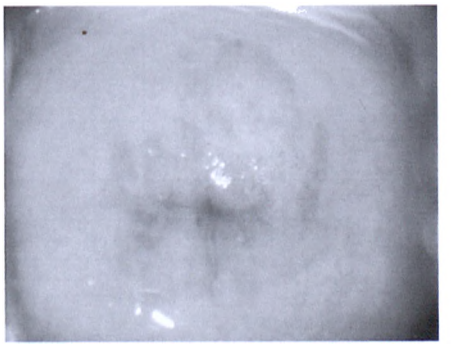
DNA HPV detection. HPV DNA was genotyped for HPV types 16, 18, 31, 33, and 45 by multiplex PCR. Each assay used 5 pmol of eluted nucleic acids and 20 pmol of each of the four primer sets. The reaction mixture contained 25 pmol of HotStart Taq master mixture (Qiagen, Germany) and 10 pmol of RNase-free water in a final volume of 50 pmol. The amplification profile consisted of 15 min at 95°C to activate the HotStar Taq DNA polymerase (Qiagen), followed by 45 cycles of denaturation (94°C for 30 s), annealing (56°C for 40 s), and extension (72°C for 40 s). Assays were performed on an iCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Amplicons were detected by electrophoresis of 20 pmol of the amplification products in a 2% agarose gel and were visualized by ethidium bromide staining under a UV light transilluminator (Fluor-S). The molecular sizes of the amplicons (for HPV type 16 [HPV-16], 152 bp; for HPV-18, 216 bp; for HPV-31, 513 bp; for HPV-33, 455 bp; and for HPV-45, 124 bp) were determined by matching them against commercial DNA molecular size markers (molecular weight markers V and VIII; Roche Diagnostics, GmbH, Mannheim, Germany).

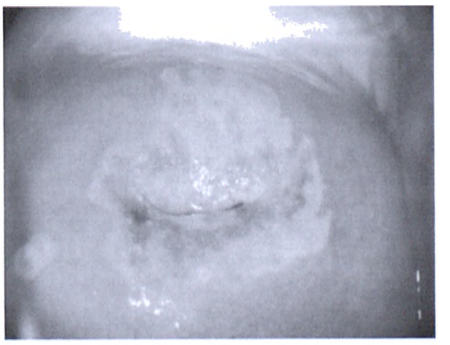
HPV mRNA detection. Samples were analyzed for HPV E6 and E7 mRNA by real-time multiplex nucleic acid sequence-based amplification. Transcripts of HR HPV types 16, 18, 31, 33, and 45 were detected by the NucliSens EasyQ HPV assay (bioMerieux, Poland), according to the manufacturer's instructions.

Statistical analysis was performed using Statistica software.- Statistica 6 PL. The Mann-Whitney U Test and student T test were performed.

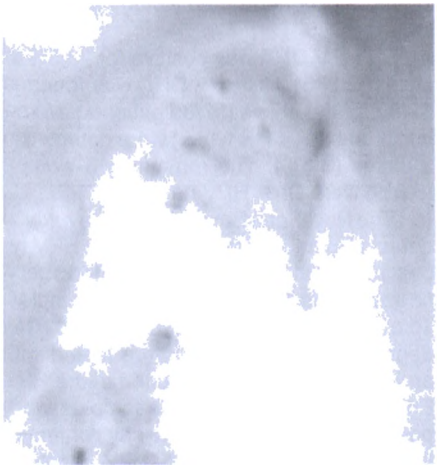


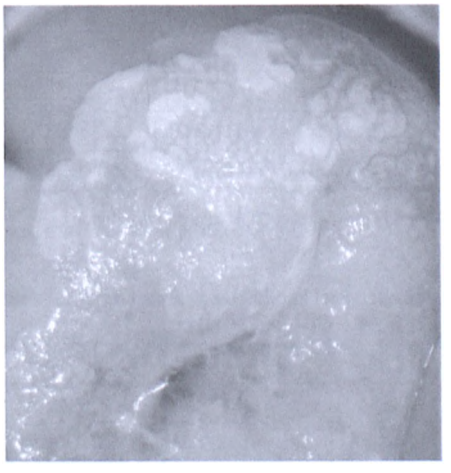
Colpophotograph 1
Typical squamous cell papilloma (characteristic papilloma surface within the transition zone).



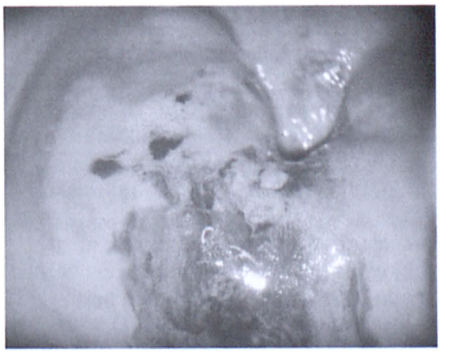


Colpophotograph 2
Image suggesting minor changes. Slight blanching after addition of acetic acid is slowly emerging and rapidly disappearing.





Colpophotograph 3
Images suggesting large degree changes. Thick dots and a wide, irregular mosaic of multipurpose- shape.



Colpophotograph 4
Image suggesting invasive cancer.
Irregular surface, erosion or ulceration.

Table IV
Prevalence of E6 and E7 transcripts in HPV types 16, 18, 31, 33 and 45.

HPV genotype mRNA	Number of patients infected with the indicated HPV type(s) as part of							
	Single infection (n=28)	Multiple infection (n=1)					Total	% of patients
		16	18	31	33	45		
16	10 (35.7%)				1 (3.57%)		11/28	39.28%
18	4 (14.28%)						4/28	14.28%
31	3 (10.7%)						3/28	10.7%
33	7 (25%)						7/28	25%
45	3 (10.7%)						3/28	10.7%

Results

Cytological colposcopical and histological findings

In a group of 85 women, an accurate view of cytology was obtained in 12 patients - average age 42.8 (range 23 to 81). The most frequent abnormal cytologic diagnosis was LSIL, confirmed in 27 (31.7%) women-mean age 36.7, range being 21 to 73.

The next diagnosis was ASCUS 30.5%, mean age 38.7, range from 22 to 59 years, diagnosis of HSIL - 18 women (21%), mean age 34.3, ranging from 19 to 60 years, and cytological diagnosis in two cases of AGC 2.35 %, mean age 45.4, range from 35 to 56 years of age. In any case, cytological diagnosis did not include squamous cell

In 5 cases, there were unsatisfactory Colposcopy images (5.8%). Satisfactory images were obtained in the remaining 92.1%. A valid colposcopic picture was found in 8.23% (occurred in 7 cases), a picture suggesting HPV infection (Fig. 1) and low-grade changes (Fig. 2) was found in 50.58% (43 cases) and 35.3% of colposcopic pictures were suggestive of high grade changes (Figure 3) and squamous cell carcinomas (Fig. 4) (30 cases), (Table II)

Because the study design included the transformation zone biopsy in all cases, in two cases, the histopathology confirmed diagnosis of cervical squamous cell carcinoma (2.3%), CIN1 in 44 cases (51.7%), CIN2 in 20 cases (2.53%), CIN 3 in 14 cases (16.4%) - Table III.

HPV DNA and mRNA detection results.

All 85 cases underwent genotyping for mRNA and DNA viruses. Transcripts E6 and E7- were found in 27 cases. Most frequently (39.28%) detected were transcripts belonging to HPV-16. The next most common (25%) was HPV-33, 4.28% of HPV-18, and 10.7% were HPV-31 and HPV-45. In one case (3.6%), coexistence of types 16 and 33 was confirmed (Table IV).

The presence of HPV DNA was detected in 77 cases (90.5%), including 20 cases (23.5%) were low-risk HPV, in 57 cases (67.05%) detected the presence of viral DNA at high risk. Mixed infection occurred in 3 cases (Table V).

In the Mann-Whitney U test comparing the relative expression of HPV mRNA, 6 parameters of risk for high grade cervical intraepithelial neoplasia were established and statistically significant differences were found for the variable cytology, colposcopy, and number of sexual partners> 5. The presence of HPV mRNA was significantly correlated with high-grade cytological changes according to the TBS classification. $p<0.001$.

However, in the Student t test, a statistically significant more frequent incidence of HPV infection (positive results of HR HPV DNA and mRNA) was observed in patients with a greater number of sexual partners, but this trend was more important for the average mRNA (4.28 at p - 0.0002). The NIR test, assuming a variable age of initiation, observed differences in the distribution of HPV mRNA transcripts depending on the age of sexual initiation (Fig. 1).

A difference in expression was observed between HPV DNA and HPV mRNA, depending on the age of sexual initiation, number of pregnancies and births, and smoking cigarettes; however, it is with the number of sexual partners that this difference reached

Table V
Results of HPV DNA testing.

Low Risk (LR) 6,11,42,43,44	20	23.5%
High Risk (HR) 16,18,31,33,35,39,45,51,52,56,58,59,68	57	67.5%
Mixed (LR+HR)	3	3.5%
Negative	5	5.8%

Table VI
The presence of HPV DNA and RNA depending on certain parameters tested
Statistically significant differences (p<0.001) were observed in the number of sexual partners in life. HPV RNA was found more often in women with more sexual partners.

	DNA	RNA	p
Mean age mean(±SD#) [years]	36.66 (±13.24)	34.74 (±10.22)	NS**
Mean age at first sexual intercose (±SD#) [years]	18.54 (±2.05)	17.89 (±1.91)	NS**
Median number of sexual partners (IQR$)	3 (2)	4 (3)	<0.001*
Median number of pregnancies (IQR$)			
Median number of deliveries (IQR$)	1 (2)	2 (2)	NS**
Smokers / Non-smokers	12 (20.0%) / 48 (80.0%)	7 (21.8%) / 25 (78.2%)	NS**

*statistically significant; **NS-statistically nonsignificant; #SD-Standard Deviation; $IQR-Interquartile Range

Table VII
The presence of HPV DNA and RNA from cytological, colposcopical and histological testing of cervical specimen.

Results of diagnostic tests	DNA	RNA	p
Pap-smear			
Normal	10 (11.7%)	2 (2.35%)	
LSIL / ASC-US	50 (50.6%)	10 (11.7%)	0,022
HSIL / ASC-H	4 (4.7%)	14 (16.5%)	
AGC / AGC-US / ACG-H	0	2 (2.3%)	
Ca	0	0	
Colposcopy			
Unsastisfactory	5 (5.9%)	0	
Normal	4 (4.7%)	3 (3.5%)	<0.001
LSIL impression	33 (38.8%)	10 (11,7%)	
HSIL impression & cancer	16 (18.8%)	15 (17,6%)	
Histologic examination			
Normal	3 (3.52%)	0	
CIN 1 + HPV	37 (43.5%)	9 (10.6%)	<0.001
CIN2 + CIN3	14 (16.5)	19 (22.3%	
Ca	0	2 (2,35%)	

statistical significance. Women with positive HPV mRNA expression had an average of four sexual partners and the women with HPV DNA three sexual partners (p <0.001).

In women who began sexual activity before 17 years of age, transcripts of HPV-31 and 45 were most frequently observed. In women beginning sexual activity after 20 years of age, mRNA transcripts of HPV types 16 and 18 were most commonly observed (Table VI)

Statistically significant differences (p <0.001) were observed in the number of sexual partners in life. HPV RNA was found more often in women with more sexual partners.

In 10 (1.7%) women with normal cytology, HPV DNA was detected, and in 2 (2.35%), HPV mRNA was detected. In the group of cytologic smears of LSIL / ASC-US, HPV DNA test was positive in 50 (50.5%) women and mRNA in 10 (11.7%) women. In the cytologic diagnoses for the group of HSIL / ASC / H, the presence of HPV DNA was found in 4 (4.7%) women and HPV mRNA in 14 (16.5%) women. In the case that cytological results for AGC / AGC-US was not HPV DNA, the HPV mRNA was positive in 2 (2.3%) women. These differences were statistically significant (p<0.22).

However, in the group of images colposcopy identified as unsatisfactory, colposcopy images were identified as positive for HPV DNA affecting five (5.9%) and therefore, all women with this colposcopy picture. In the group of normal colposcopy images, positive HPV DNA test was confirmed in four (4.7%) women and the presence of mRNA transcripts E6 and E7 were found in 3 (3.5%) women. In contrast, if a group of colposcopy images suggested final histological diagnosis of LSIL, presence of HPV DNA was found in 33 (38.8%) women and HPV mRNA in 10 (11.7%) women. In the group of images suggesting a final diagnosis of HSIL or cervical cancer, HPV DNA was positive in 16 (18.8%) women and HPV mRNA in 15 (17.6%) women. The obtained results differed significantly (p<0.001).

HPV DNA and mRNA tests. All cervical specimens were tested for HPV DNA and E6/E7 HR HPV - mRNA namely 16,18,31,33 and 45. The tests were performed by investigators blinded to the cytology and histology results.

LR HPV DNA was positive for 23,5% of cases (20/85). HR HPV DNA was found in 67,5% of the cases (57/85 patients). Mixed infections (LR + HR types) were detected in 3 cases (3.5%). The remaining 5 women (5.8%) were DNA HPV negative.

The NucliSens EasyQ HPV assay, which detects E6 and E7 mRNA from HR HPV types 16, 18, 31, 33, and 45, identified specific transcripts in 28 of 85 (32.94%) samples. To avoid false-negative results due to RNA degradation, all samples were tested with an RNA control (U1A) included in the HPV E6 and E7 mRNA test. All samples were positive. In each case, the assay confirmed the presence of the specific HPV genotype previously revealed by the RNA-based method. The most common HPV genotype revealed by RNA testing was HPV-16 (10/28 cases [35.7%]), followed by HPV-33 (7/28 cases [25%]), HPV-18 (4/28cases [14.28%]), HPV-31 (3/28 cases [10.7%]), and HPV-45 (3/28 cases [10.7%]). In 27/28 cases (96.4%), the test detected infections with single genotypes; 1/28 cases (3.5%) involved infections with multiple genotypes namely HPV 16 and HPV 33 (Table II).

The final result of histological examination for diagnosis of normal epithelium identified the presence of HPV DNA in 3 (3.52%) women. In this group, the results of the HPV mRNA test was negative. The group with CIN1 diagnosis tested positive for HPV DNA in 37 (43.5%) women and HPV mRNA in 9 (10.6%). In the group of diagnoses with CIN2 / 3, the presence of HPV DNA involved 14 (16.5%) women and HPV mRNA in 19 (22.3%) women. In 2 (2.3%) cases of squamous cell carcinoma of the cervix, the presence of HPV mRNA transcripts E6 and E7 was confirmed, while there was no presence of HPV DNA. The resulting differences achieved statistical significance (p<0.001). (Table VII)

The Gamma correlation coefficient for colposcopy and histopathology final result was G1 = 0.825. For colposcopy combined with the assessment of expression of HPV E6/E7 mRNA with the final result of the histopathological examination, Gamma correlation coefficient was G2 = 0.947 at the significance level of p<0.001.

Therefore, the coefficient of G2 is significantly higher than the G1. It follows that combining colposcopy assessment with HPV mRNA testing is more consistent with

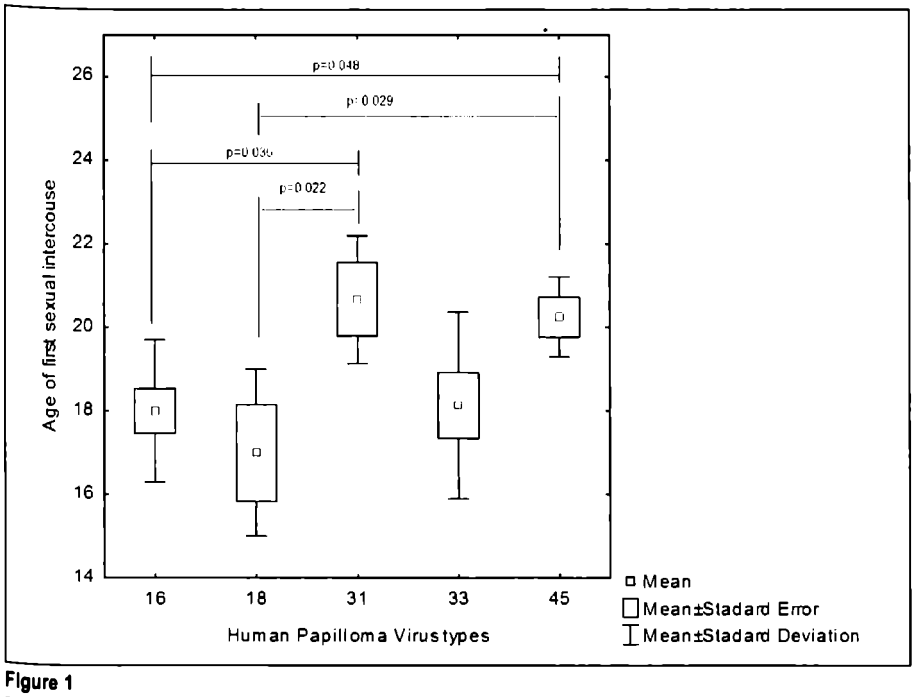


Figure 1
The relationship between age of starting sexual activity and the types of HPV mRNA expressed.

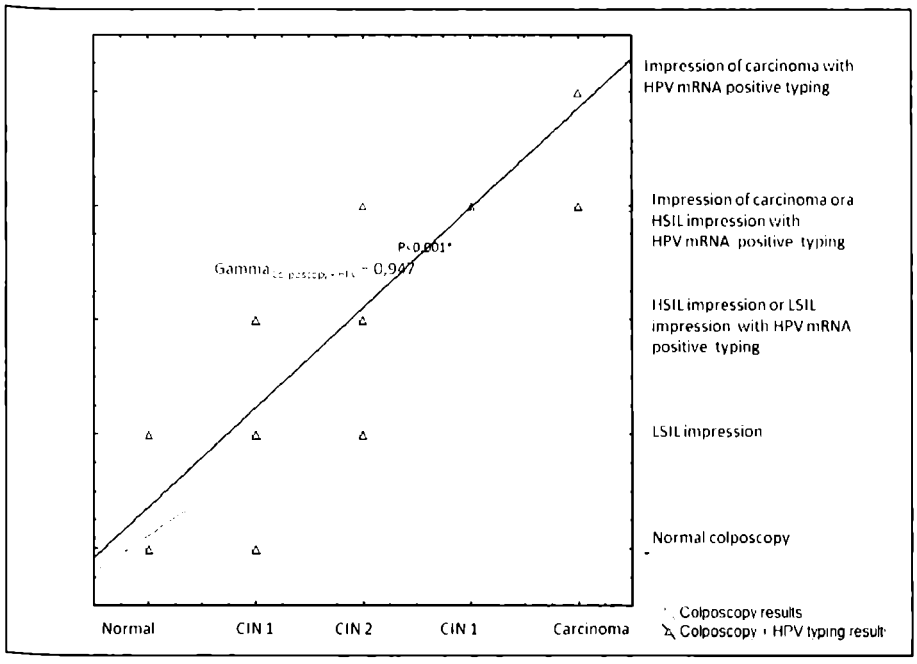


Figure 2
Graph showing the relationship between the result of colposcopy (colposcopic impression) and the final result of histological examination, and between the result of colposcopy (colposcopic impression) combined with the assessment of HPV mRNA and the final result of histological examination.

the final result of histological examination. (Figure 2)

The recognition of HPV mRNA does not change the correlation between the cytological test and the histopathological examination results, whereas it increases consistency between the result of colposcopy and histopathology. This relationship is statistically significant ($p < 0.001$).

Discussion

Cervical cancer affects about 500,000 women worldwide annually and causes about 250, 000 deaths. It is the second most common cancer among women. In virtually every case of cervical cancer (99.7%), a viral genetic material with a high potential of HPV oncogenesis has been found (IARC 2010).

Currently, strategies to combat cervical cancer occurs in two stages: by prophylactic vaccination against certain types of HPV, and through cytological screening. Unfortunately, vaccination does not protect against all types of infection with highly oncogenic HPV, and therefore, did not constitute full protection against the development of cervical cancer. High costs are a barrier to preventive vaccination and against the introduction of universal vaccination programs in developing countries where the incidence of cervical cancer is highest. In developing countries, the incidence of cervical cancer is maintained at a very low level, with screening by cervical cancer cytological tests. In the course of cytological screening, the cytological abnormalities in cells taken from the surface of the cervix are detected using cytology. Cytological screening is effective in reducing the incidence of cervical cancer in countries such as Poland and the United Kingdom, However, this requires a network of highly-trained cytologists and the percentage of false negative Pap smears is high (reaching 20%). In Western countries, vaccinations are carried out along with cytologic screening (since 2008).

It is widely believed that these immunizations significantly reduce the incidence of infections with certain types of high oncogenic potential HPV in the general population, and therefore, in the near future, you will need a faster, less expensive and also more effective model of screening for cervical cancer [2,15,17,20]

Bearing in mind that according to data from the NCI and IARC, HPV DNA prevalence among women with normal cytology results ranges of 7.9% in Europe to 22.1% in Africa.

In many countries, clinical trials and screening pilot programs using HPV DNA have been underway. In the U.S., for example, HPV DNA testing is a supplement to cytological studies in women over 30 years of age. This study has higher sensitivity and high negative predictive value (NPV) compared with cytology (> 99% vs 96%). However, the sensitivity of HPV DNA in comparison with cytology is 96-98% vs. 53 to 73% (6.34). Currently, in the U.S., the only authorized clinical test for HPV testing is Capture2 Hybrid (HC2), which detects the presence of one or more of 13 oncogenic HPV types. A positive result should be the result of greater than 1.0 RLU (relative light unit), compared to 1 pg / ml HPV DNA from the control sample. HC2 is currently used in two cases, namely, as a second test in the case of abnormal Pap smear-ASCUS and in primary screening for cervical cancer in combination with cytology smear for women over 30 years of age [28].

Although the sensitivity of testing for HR HPV DNA for discovering CIN and cervical cancer is higher than cytology, its specificity is lower which leads to low positive predictive value (PPV) for changes in the type of CIN2 +. This leads to unnecessary biopsy of the cervix in all too many women [33].

Recent years have brought a new, promising diagnostic potential: diagnosis of persistent HR HPV infection without the need for HPV genotyping twice at an interval of 6-12 months. Currently, there are tests indicating material obtained in the presence of cervical HR HPV mRNA. This technique is based on the detection of mRNA gene transcripts E6 and E7, HPV 16, 18, 31, 33 and 45.

A positive result of the test not only allows for identification of the presence of one or more of the most frequent types of high-risk HPV, but considers also, that viral genes rewrite in surrounding keratinocytes and oncoprotein synthesis is the earliest stage of recognizing the process of carcinogenesis of the cervix. Many clinical studies [2,14,15,17,20,22-24,38] attributed this

molecular test to have twice the positive predictive value (PPV) compared to the classic methods of detection of HPV DNA-based hybridization technique and polymerase chain reaction. According to Molden et al., PPV for the test was based on the detection of E6 and E7 mRNA, and was used to detect changes in CIN2-3 -and was 37.5% compared to 15.4% for DNA detection [23].

Many researchers and scientific societies (ACOG - American College of Obstetricians and Gynecologists) have started using molecular diagnosis of HPV as a preliminary test, whose results will form the basis of qualifications for cytological smear. Another argument for this screening procedure would be a higher sensitivity and negative predictive value (NPV - negative predictive value) of HPV molecular diagnostics in comparison to the higher specificity and positive predictive value in cytodiagnostics.

The widely accepted model of the disease coupled with the latest scientific data show that in low-grade tumor markers of E7 are expressed in the lower layers of the epithelium. The onset of E4 expression occurs in place of decreasing expression of E7. After the loss of E7 expression, E4 begins to accumulate. The "switch" in expression from E7 to the expression of E4 is correlated with amplification of the genome and represents a key event leading to virion attachment in the upper layers of the epithelium. In the progression of neoplasia, E7 is expressed from the basal membrane to upper layers of the epithelium, and the amount of E7 protein in the cell is increased [28,29].

This de-regulation of gene expression does not always lead to the production of virus but is a key event in the progression of low grade cancer.

Cervical intraepithelial neoplasia is usually treated by surgical excision of the transformation zone of the cervix. Such a procedure may increase the risk of preterm delivery and perinatal mortality increases [8-10,31].

Therefore, we conducted a clinical study, in attempt to validate the determination of HPV mRNA in the daily practice of colposcopy. Selection of the study group support the conclusion that the study group has been selected in accordance with the rules in an optimal manner. The study included 85 women presenting to a clinic Cervical Pathology Jagiellonian University in Krakow.

A satisfactory colposcopic picture was obtained in 80 (94.2%) women. Among these images, in 43 (50.58%) cases there was a group of images suggesting low-grade changes - colposcopic impression LSIL, whereas in 30 (35.29%) women were images suggesting colposcopic high grade changes and/or cancer of the cervix (colposcopic impression HSIL / cervical cancer). Thus the distribution of colposcopy images should be considered in line with the results of other authors [36]. In Krakow, colposcopic testing is an integral part of the pelvic examination and can be evaluated and classified independently of the biopsy from the cervix. Nevertheless, the present study included only cases in which the colposcopic study was verified with histological results.

In the Mann-Whitney U analysis comparing HPV mRNA, 6 parameters with the risk of high grade cervical intraepithelial neoplasia with statistically significant differences were found for the variable cytology, colposcopy and number of sexual partners> 5. The presence of HPV mRNA was significantly correlated with high-grade cytological abnormalities in the TBS classification.

However, when it comes to the Gamma correlation coefficients, they amount to colposcopy and the final result of the histopathological examination G1 = 0.825, and for colposcopy combined with the assessment of expression of HPV E6/E7 mRNA with the final result of the histopathological examination, G2 = 0.947 at the level of statistical significance $p<0.001$; that is, the coefficient G2 is significantly higher than the G1.

Therefore, the presence of transcripts E6 and E7 may be a specific marker of high-grade changes, a positive RNA test is characterized by a higher predictive value than the evaluation of HPV DNA testing.

Our results confirm the findings of other authors [21,37,38], but for the first time in our material, we have shown that adding the HPV mRNA test to diagnostics increases the correlation of colposcopy results with the final histopathological examination results.

Determination of HPV mRNA does not affect the correlation between the result of a cytological test and the ultimate result of histopathological examination, while increasing consistency between the result of colposcopy and histopathology.

A lower correlation coefficient was observed in the result of cytological examination combined with assessment of expression of HPV mRNA transcripts to the final result of histological examination, in comparison to the result of cytology with the final result of histological examination. This reflects the transitional nature of HPV infection but it may also provide for detection of chronic HPV infection in the absence of morphological markers of cervical intraepithelial neoplasia and the detection of the first molecular phase of carcinogenesis within the cervical epithelium [31]. Evaluation of human papillomavirus protooncogene expression statistically confirms the impact of overexpression of E6 and E7 and E4 on the occurrence of cervical intraepithelial neoplasia in CIN2/3), [1,9,10]. Moreover, the possibility that women are HPV DNA positive but with a negative result for HPV mRNA can not rule out that these results will never progress to invasive cervical cancer.

The continuous expression of viral oncogenes E6 and E7 is a necessary step in the process of carcinogenesis [42]. Therefore, the identification of E6 and E7 HPV mRNA demonstrating high oncogenic potential, can also be a marker of infection-initiated carcinogenesis of the cervix. HPV mRNA testing is characterized by a higher specificity in detecting high-grade epithelial changes compared with HPV DNA testing. [12,16,27].

This increase in specificity may be particularly important for younger women, among whom prevalence of HPV infection is particularly high. This study [22] of women before age 30 whereby in 14.5%, the presence of HPV mRNA was confirmed using the HPV proofer PreTect compared to 20.8% positive results in the study of HPV DNA PCR. In the same cohort, abnormal cytology was found in 2.8% of women [21]. The results of these studies confirm the value of applying HPV mRNA assessment results to cases where HPV DNA results may also be available.

In a study, Cushieri et al., [5], including HPV positive women with normal cytology, presence of mRNA transcripts of E6 and E7 HPV was less sensitive but a more specific test in the observational study to detect high-grade epithelial changes. More often, it was also associated with the presence of chronic infection with HPV. Another potential use of HPV mRNA testing may be the result of women with equivocal Pap smear. Among women diagnosed with ASCUS, 21% were positive in the mRNA test vs. 25% positive for HPV DNA test. For women diagnosed with LSIL, percentages were respectively 30 and 50%.

HPV RNA testing may also have the potential use as a primary screening tool. But so far, there is no research directly comparing the sensitivity and specificity of this test among women with normal Pap smear results. In particular, the loss of specificity due to the detection of only five high-risk HPV types requires further evaluation in the initial screening and triage. We are currently working on a test assessing a broad spectrum (15 types) HPV mRNA (GenProbe).

References

1. **Basta A.**: Znaczenie infekcji wirusowej HPV oraz obniżonego poziomu witaminy A w surowicy krwi jak i niektórych czynników osobniczych i środowiskowych w morfogenezie raka szyjki macicy. Rozporawa habilitacyjna, Kraków 1990.
2. **Christopherson W.M., Lundin F.E. Jr, Mendez W.M. et al.**: Cervical cancer control: a study of morbidity and mortality trends over a twenty-one-year period. Cancer 1976, 38, 1357.
3. **Clifford G.M., Smith J.S., Plummer M. et al.**: 2003. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br. J. Cancer 2003, 88, 63.
4. **Coquillard G., Palao B., Patterson B.**: Quantification of intracellular HPV E6/E7 mRNA expression increases the specificity and positive predictive value of cervical cancer screening compared to HPV DNA. Gynecol. Oncol. 2011, 120, 89.
5. **Cuschieri K.S., Whitley M.J., Cuble H.A.**: Human papillomavirus type specific DNA and RNA persistence--implications for cervical disease progression and monitoring. J. Med. Virol. 2004, 73, 65.
6. **Cuzick J., Szarewski A., Cuble H. et al.**: Management of women who test positive for high - risk types of human papillomavirus, The HART study. Lancet 2003, 362, 1871.
7. **Ding J., Doorbar J., Li B. et al.**: Expression of papillomavirus L1 proteins regulated by authentic gene codon usage is favoured in G2/M-like cells in differentiating keratinocytes. Virology 2010, 399, 46
8. **Doorbar J., Myers G.**: The E4 protein. In "Human Papillomaviruses 1996" (G. Myers, H. Delius, J. Icenogel, H.-U. Bernard, C. Baker, A. Halpern, and C. Wheeler, Eds.), Vol. III, pp. 58-80. Los Alamos National Laboratory, Los Alamos, New Mexico, USA
9. **Doorbar J., Elston R., Napthine S et al.**: The E1^E4 protein of human papillomavirus type 16 associates with a putative RNA helicase through sequences in its C terminus. J. Virol. 2005, 74, 2005, 10081.
10. **Doorbar J., Foo C., Coleman N et al.**: Characterisation of events during the late stages of HPV16 infection in vivo using high affinity synthetic Fabs to E4. Virology 1997, 238, 40.
11. **Falcinelli C., Claas E., Kleter B. et al.**: 1992. De-

tection of the human papillomavirus type 16 mRNA-transcripts in cytological abnormal scrapings. J. Med. Virol. 1992, 37, 93.
12. **Keegan H., Mc Inerney J., Pilkington L. et al.**: Comparison of HPV detection technologies: hybrid capture 2, PreTect HPV-Proofer and analysis of HPV DNA viral load in HPV 16, HPV18 and HPV33 E6/E7 mRNA positive specimens. J. Virol. Meth. 2009, 155, 61.
13. **Hsu E.M., McNicol P.J., F. B. Guijon F.B. et al.**: 1993. Quantification of HPV-16 E6-E7 transcription in cervical intraepithelial neoplasia by reverse transcriptase polymerase chain reaction. Int. J. Cancer 1993, 55, 397.
14. **Jeon S., B. L. Allen-Hoffmann B.L., Lambert P.F.**: Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. J. Virol. 1995, 69, 2989.
15. **Johannesson G., Geirsson G., Day N.**: The effect of mass screening in Iceland, 1965-74, on the incidence and mortality of cervical carcinoma. Int. J. Cancer 1978, 21, 418.
16. **Kraus I., Molden T., Erno L.E. et al.**: Human papillomavirus oncogenic expression in the dysplastic portio; an investigation of biopsies from 190 cervical cones. Br. J. Cancer 2004, 90, 1407.
17. **Lăără E., Day N.E., Hakama M.**: Trends in mortality from cervical cancer in the Nordic countries: association with organised screening programmes. Lancet 1987, 1 (8544), 1247.
18. **Lazo P. A.**: Papillomavirus integration: prognostic marker in cervical cancer. Am. J. Obstet. Gynecol. 1997, 176, 1121.
19. **Meijer C.J., A. J. Van Den Brule A.J., Snijders P.J. et al.**: Detection of human papillomavirus in cervical scrapes by the polymerase chain reaction in relation to cytology: possible implications for cervical cancer screening. IARC Sci. Publ. 1992, 119, 271.
20. **Miller A.B., Lindsay J., Hill G.B.**: Mortality from cancer of the uterus in Canada and its relationship to screening for cancer of the cervix. Int. J. Cancer 1976, 17, 602.
21. **Molden T., Kraus I., Karlsen F. et al.**. Human papillomavirus E6/E7 mRNA expression in women younger than 30 years of age. Gynecol. Oncol. 2006, 100, 95.
22. **Molden T., Kraus I., Karlsen F., et al.**: Comparison of human papillomavirus messenger RNA and DNA detection: a cross-sectional study of 4,136 women >30 years of age with a 2-year follow-up of high-grade squamous intraepithelial lesion. Cancer Epidemiol. Biomarkers Prev. 2005, 14, 367.
23. **Molden T., Kraus I., Skomedal H. T. et al.**. PreTect HPV-Proofer: real-time detection and typing of E6/E7 mRNA from carcinogenic human papilloma-viruses. J. Virol. Methods 2005, 142, 204.
24. **Molden T., Nygard J.F.,Kraus I. et al.**: Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV-Proofer and consensus PCR: a 2-year follow-up of women with ASCUS or LSIL Pap smear. Int. J. Cancer 2005, 114, 973.
25. **Munoz N., Castellsague X., Berrington de Gonzalez et al.**: HPV in the etiology of human cancer. Vaccine 2006, 24 (Suppl. 3), 1.
26. **Munoz N., Bosch F.X., de Sanjosé S. et al.**: and the International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N. Engl. J. Med. 2003, 348, 518.
27. **Nakagawa S., Yoshikawa H., Yasugi T. et al.**: Ubiquitous presence of E6 and E7 transcripts in human papillomavirus-positive cervical carcinomas regardless of its type. J. Med. Virol. 2000, 62, 251.
28. **New Device Approval**: Digene Hybrid Capture 2 High-Risk HPV DNA Test - P890064 S009 A004 . Rockville, Md: U.S. Food and Drug Administration, Center for Devices and Radiological Health, 2003
29. **Ostör A.G.**: Natural history of cervical intraepithelial neoplasia: a critical review. Int. J. Gynecol. Pathol. 1993, 12, 186.
30. **Cattani P., Zannoni G.F., Ricci C. et al.**: Clinical performance of human papillomavirus E6 and E7 mRNA testing for high-grade lesions of the cervix. J. Clin. Microbiol. 2009, 47, 3895.
31. **Peh W.L., Middleton K., Christensen N. et al.**: Life cycle heterogeneity in animal models of human papillomavirus-associated disease. J. Virol. 2002, 76, 10401.
32. **Petry K.U., Menton S., Menton M. et al.**: Inclusion of HPV testing in routine cervical cancer screening for women above 29 years in Germany: results for 8466 patients. Br. J. Cancer 2003, 88, 1570.
33. **Ronco G., Giorgi-Rossi F., Carozzi F. et al.**: Results at recruitment from a randomized controlled trial comparing human papillomaviruss testing alone with conventional cytology as the primary cervical cancer screening test. J. Natl. Cancer Inst. 2008, 100, 492.
34. **Sankaranrayanan R., Budukh A., Rajkumar R.**: Effective screening programmes for cervical cancer in low- and middle- income developing countries. Bull World Health Organization 2001, 79, 954.
35. **Schwarz E., Freese U.K., Gissmann, L. et al.**: 1985. Structure and transcription of human papillomavirus type 18 and 16 sequences in cervical carcinoma cells. Nature 1985, 314, 111.
36. **Shafi M.I., Luesley D.M.**: Management of low grade lesions: Follow-up or treat? Baillieres Clin. Obstet. Gynaecol. 1995, 9, 121.
37. **Shah S.D., Doorbar J., Goldstein R.A.**: Analysis of host-parasite incongruence in Papillomavirus evolution using importance sampling. Mol. Biol. Evol. 2010 27, 1301.
38. **Sotlar K., Selinka H.C., Menton M. et al.**: Detection of human papillomavirus type 16 E6/E7 oncogene transcripts in dysplastic and non dysplastic cervical scrapes by nested RT-PCR. Gynecol. Oncol. 1998, 69, 114.
39. **Szarewski A., Ambrosine L., Cadman L. et al.**: Comparison of predictors for high- grade cervical intraepithelial neoplasia in women with abnormal smears. Cancer Epidemiol. Biomarkers Prev. 2008, 17, 3033.
40. **Waggoner S.E.**: Cervical cancer. Lancet 2003, 361, 2217.
41. **Zheng T., Holford T.R., Ma Z.Y. et al.**: The continuing increase in adenocarcinoma of the uterine cervix: a birth cohort phenomenon. Int. J. Epidemiol. 1996, 2, 252.
42. **zur Hausen H., de Villiers E.M.**: Human papillomaviruses. Ann. Rev. Microbiol. 1994, 48, 427.
43. **zur Hausen H.**: Papillomavirus infections: a major cause of human cancers. Biochim. Biophys. Acta 1996, 1288, F55.
44. **zur Hausen H.**: Papillomavirus and cancer: from basic studies to clinical application. Nat. Rev. 2002, 2, 342