Human Papillomavirus Infection in Cervical Carcinoma

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The aim of this study was to detect human papillomavirus (HPV) in healthy women and in women with cervical cancer by means of DNA hybridization (Hybrid Capture II) and to identify HPV types using polymerase chain reaction with type-specific primers (TS-PCR). The study included 100 cervical carcinoma patients (92 – squamous cell carcinoma and 8 – adenocarcinoma or adenosquamous carcinoma). Hybrid Capture II (HCII) and polymerase chain reaction with type-specific primers (TS-PCR) were used for human papillomavirus (HPV) detection and typing. Among 100 cervical carcinoma patients 97 were positive for HPV infection (HC II was used): 89 (92%) were positive for high-risk group viruses, 8 (8%) – for low- and high-risk group viruses. In a control group of 100 women 26 were positive for HPV infection: 4 (15%) – for low-risk group viruses, 14 (54%) – for high-risk group and 8 (31%) – for low- and high-risk group viruses. After HPV typing (TS-PCR was used) in patients with cervical carcinoma the most frequently type was HPV-16: this type was found in 51 patients (52%). In control group the most frequent type was HPV-6/11: this type was found in 8 women (31%). The HC II system was developed for routine HPV detection in cervical scrapes, but this system detects 18 anogenital HPV types with a sensitivity that is slightly below that of the commonly used general-primer PCR or TS-PCR techniques test.

Key words: cervical carcinoma, HPV, Hybrid Capture II, type-specific PCR

INTRODUCTION

Worldwide, cervical cancer is the second most common cancer affecting women. Although screening programs to identify precursor lesions of cervical cancer have reduced significantly the mortality and morbidity of this disease, 500 000 new cases of invasive cancer of the cervix are diagnosed annually (1). The increase of cervix cancer incidence and especially mortality from this disease is a major problem for Lithuania. In 1999 diagnosed were 461 and in 2000 – 468; new cases of cervical carcinoma, the morbidity rate of this pathology in 1999 was 23.6/100,000 and in 2000 – 24.0/100,000 (2, 3).

The age-standardised incidence rates (ASR World) varied in Lithuania from 12.9 to 15.8 during the period 1983–1997. These rates are similar to the rates observed in the neighbouring countries (period 1988–1992): Estonia 14.1, Poland, Warsaw city 15.2. These rates are significantly greater than in most developed countries with well-organised screening programs (in Finland ASR World is about 3.0). Moreover the incidence rates have increased annually by 3.3% with a 95% confidence interval (Cl 95% 2.2–4.4) among younger women (30–49). Presently, upward trends in cervical cancer incidence among young women exists in some other countries, but the increase in Lithuania is one of the greatest (4).

Many studies that have focused on the risk factors of cervical cancer identification have shown that cervical neoplasia is associated with prevalence of human papillomaviruses (HPV) (5–7). Now there is no doubt as to the important aetiological role of HPV in cervical carcinogenesis. The characterisation of new genital HPV types and the development and application of highly sensitive HPV detection systems have shown that nearly 100% of all cervical squamous cell carcinomas (SCCs) and more than 70% of cervical adenocarcinomas (ADs) are associated with HPV (8). HPV 16 is the most frequent type in SCCs. More than 50% of these tumours

worldwide harbour HPV 16 DNA (8, 9). In contrast, more than 50% of cervical ADs are associated with HPV 18 (10). The aim of this study was to detect human papillomavirus (HPV) and cancerogenic types in healthy women and in women with cervical cancer using DNA hybridization (Hybrid Capture II) and to identify HPV types using polymerase chain reaction with type-specific primers (TS-PCR).

MATERIALS AND METHODS

Subjects and data collection. 100 women with cervical cancer were examined: 92 – with SCC, 6 – with AD and 2 – with adenosquamous cell carcinoma (AD/SCC). Pathologists of the national Pathology Centre confirmed the diagnosis cytologically and histologically.

Table 1 shows the distribution of cases according to FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) and TNM.

Poorly differentiated cervical carcinomas were predominant (Table 2).

One hundred healthy volunteer women as control subjects served. The subjects were aged between 19 to 81 years (3). Table 3 shows the distribution of cases and controls in relation to age.

Brush swabs were used to obtain the endocervical samples. The material from the brush was collected in 1 ml transport medium of Digene diagnostics. These samples were tested for HPV DNA by HC II and HPV types by TS-PCR. Before testing all samples were stored at –20 °C.

HPV DNA detection and typing. Cervical samples were tested for HPV DNA "low-risk " (6, 11, 42, 43, 44) and "high-risk" (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) groups by Digene HPV DNA test: HCII.

For TS-PCR HPV DNA was purified using Genomic DNA Purification Kit (Fermentas Ltd., Lithuania).

200 µl of cell sample was mixed with 400 µl of lysis solution and incubated at 65 °C for 5 min. After incubation 600 µl of chloroform was added

Table 3. **Distribution of cases and controls according to age**

Age (years)	Cases, %	Controls, %
< 20	–	5
20–29	4	41
30–39	14	29
40–49	31	15
50–59	27	8
60–69	21	2
> 70	3	–

Table 1. **Distribution of cases according to FIGO and TNM classification**

FIGO	TNM	Clinical characterization	Number of cervical cancer patients
I	T1	Cervical carcinoma confined to uterus corpus	–
I A	T1a	Invasive carcinoma diagnosed only by microscopy	1
I B	T1b	Clinically visible lesion confined to cervix	5
II	T2	Tumour invades beyond uterus but not to pelvic wall or to lower third of the vagina	–
II A	T2a	Without parametrial invasion	5
II B	T2b	With parametrial invasion	33
III	T3	Tumour extends to pelvic wall and/or involves lower third of vagina and/or causes hydronephrosis or non–functioning kidney	–
III A	T3a	Tumour involves lower third of vagina, no extension to pelvic wall	3
III B	T3b	Tumour extends to pelvic wall and/or causes hydronephrosis or non-functioning kidney	52
IV A	T4	Tumour invades mucosa of bladder or rectum and/or extends beyond pelvis	1

Table 2. **Distribution of cases according to histopathological grading**

Histopathological grading	Number of cervical cancer patients
Well differentiated, G1	3
Moderately differentiated, G2	20
Poorly differentiated, G3	77

immediately. After mixing by inversion, the samples were centrifuged at 14,000 rpm for 5 min. After centrifugation the upper aqueous phase containing DNA was transferred to a new tube. 800 µl of freshly prepared precipitation solution was added, mixed gently by several inversions at room temperature for 1–2 min and centrifuged at 14,000 rpm for 5 min. Supernatant was removed completely and DNA pellet was dissolved in 100 µl of 1.2 M NaCl solution by gentle vortexing and precipitated again by adding 300 µl of cold ethanol and keeping for 20 h at –20 °C. DNA was spun down (14,000 rpm, 8 min), the pellet was washed once with 70% cold ethanol and DNA was dissolved in 100 µl of sterile deionised water by gentle vortexing.

For HPV typing the primer pair showed in Table 4 was used (11).

TS-PCR was performed in 50 µl of PCR solution using a Taq DNA Polymerase and dNTP Set (Fermentas Ltd., Lithuania). PCR contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl 0.08% Nonidet P40, 1.5 mM $MgCl_2$, 200 nM of each dNTP, 50 pmol of each primer, 0,5 unit of Taq DNA Polymerase (recombinant). PCR was performed in a personal cycler (Eppendorff, Germany) starting from the initial denaturation step at 95 °C for 3 min followed by 40 cycles of denaturation step at 95 °C for 1 min, primer annealing step at 55 °C for 1 min 30 s and a chain elongation step at 72 °C for 1 min. A final extension of 10 min at 72 °C was used (19). In addition to HPV, all samples were tested for β globine gene by PCR. Each PCR experiment was performed with positive and negative PCR controls. For positive controls we used SiHa and HeLa cells (for HPV16 and HPV18) and for negative control samples without DNA were used. PCR products were analysed by electrophoresis in 2% of agarosis gel stained by ethidium bromide (Figure).

Aliquots (10 µl) of each TS-PCR product were resolved by electroforesis in a 2% agarose gel and stained with ethidium bromide. For TS-PCR, HPV 6/11, 16 and 18 specific primers were used. HPV 6/11 amplimer length was 140 bb, HPV 16 – 120 bp and HPV 18 – 113 bp. SiHa cells were used for the positive HPV 16 control, HeLa cells – for the HPV 18 positive control. As negative control were used specimens with deionised water (without DNA). 50 bp DNA marker was used.

Table 4. **Primer sequences used for HPV testing**

TARGET	PRIMER	SEQUENCE (5'→3')	AMPLIMER LENGTH (bp)
HPV 6/11	6–1	5'→GCTAATTCGGTGCTACCTGT	140 bp
	6–2	5'→CTTCCATGCATGTTGTCCAG	
HPV 16	16–1	5'→TCAAAAGCCACTGTGTCCTG	120 bp
	16–2	5'→CGTGTTCTTGATGATCTGCA	
HPV 18	18–1	5'→ACCTTAATGAAAAACGACGA	113 bp
	18–2	5'→CGTCGTTGGAGTCGTTCCTG	
β-Globin	PC03	5'→ACACAACTGTGTTCACTAGC	110 bp
	PC04	5'→CCACTTGCACCTACTTCAAC	

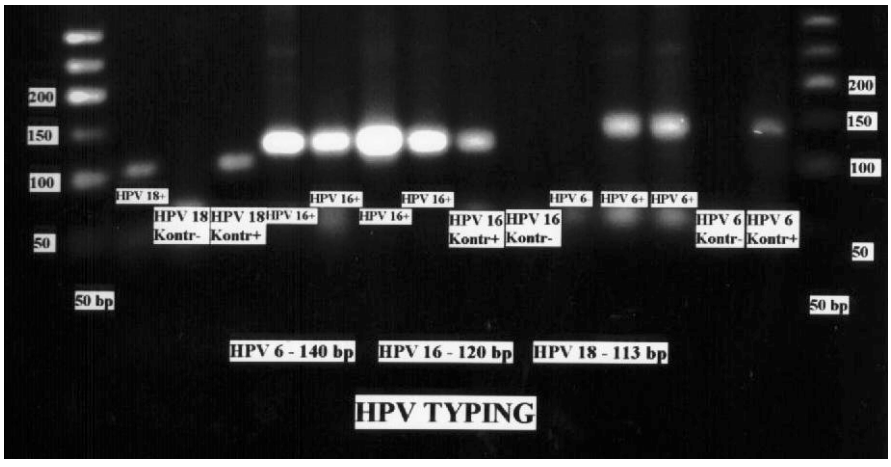

Figure. HPV typing using TS-PCR. Electrophoresis in 2% agarosis gel

RESULTS AND DISCUSSION

During the study period (2000–2001), 100 patients with cervical cancer were included in the study. 100 healthy volunteer women were identified as potential control. The study protocol was approved by the Ethical Committee of the Lithuanian Oncology Centre.

Among 100 cervical cancer patients and 100 healthy volunteer women, 97 and 26 respectively were HPV DNA positive (HC II method). "High-risk" HPV types were predominant: 92% in cancer patients and 54% in healthy women (Table 5).

After performing HPV typing (TS-PCR for HPV 6/11, 16, 18) HPV was detected in 99% of cases, because in 2 cases of ADs which were negative while testing by HC II method, HPV 16 and HPV 18 by TS-PCR were identified (Table 6).

Table 5. **Distribution of cases and controls according to the prevalence of HPV**

HPV	Cases			Controls number, %	OR	95% CI
	number		number, %			
	SCCs	ADs	total			
"Low risk"	–	–	–	4 15%		
"High risk"	85	4	89			
			92%	14 54%	49.7	21.4–116
"Low and	7	1	8	8 31%	1.00	0.360–2.78
high risks"			8%			
Negative test	–	3	3	74		

SCCs – squamous cell carcinoma, ADs – adenocarcinoma.

Table 6. **HPV results using two methods – HC II and TS-PCR**

Diagnosis	HC II	TS-PCR HPV-6/11,16 or 18	TS-PCR HPV-X	Number of cases
SCCs	+	+	–	54
	+	–	+	38
ADs	+	+	–	3
	+	–	+	2
	–	+	–	2
	–	–	–	1

SCCs – squamous cell carcinoma, ADs – adenocarcinoma

In total, among all investigated specimens of cervical cancer only one case was negative.

HPV 16 was predominant (52% of HPV-positive cases) in SCCs. Thirty-eight cases of SCCs were positive for HPV, but the type could not be identified (HPV X) (Table 7). Women positive for HPV X were also at a high risk of cervical cancer. Other countries also reporting high frequencies of HPV 16 among cervical cancer patients are Poland (78.3%), Germany (76.5%), Thailand (59.3%), India (64.0%), whereas the prevalence of HPV 16 is lower in African countries (9). Studies on HPV and cervical ADs are scarce, and the most consistent finding has been the predominance of HPV 18. Therefore, the small number of our observation limits the interpretation.

The prevalence of HPV infection was also high among healthy women (n = 100). HPV DNA was detected by HC II method in 26% of control cases. HPV 6/11 was the predominant type (31% of positive control cases). It is important to note that high-risk HPV by HC II has been detected in 22% of all investigated healthy women.

The high-risk HPV (as determined factor of cervical cancer) were detected in a number of healthy women participating in the control group of our study. This emphasizes that the development of a cervix pathology screening program is the priority in health control organization.

For clinical applications, HPV tests are useful and valuable if the HPV detection system is highly sensitive and specific, and if false-positive and false-negative results can be largely excluded. Two test systems fulfil this need at present: the Hybrid Capture II (HC II) analysis and the polymerase chain reaction (PCR). Other HPV detection methods are restricted to certain research applications (RNA *in situ* hybridisation), too complicated for routine purposes (Southern-blot hybridisation) or not sufficient in sensitivity and specificity (Dot-blot hybridisation, filter *in situ* hybridisation, DNA *in situ* hybridisation). Yet, for research applications, *in situ* hybridisation using HPV-specific DNA or RNA probes on paraffin section can be a valuable tool to study the exact localisation of HPV infection or the expression of HPV oncogenes (12, 13).

In order to detect a broad spectrum genital HPV types, many laboratories performing HPV PCR use one of the two general primer pairs: primers GP5+/GP6+ which span a region of 140–150 bp from the L1 open reading frame (14), or the primer pair MY09/MY11, which gives rise to a PCR product of approxima-

Table 7. **HPV typing using TS-PCR**

HPV type	Cervical cancer patients			Healthy women	OR	95% CI
	SCCs	ADs	total			
HPV 6/11	–	–	–	8		
HPV16	48	3	51	2	13.3	2.98–59.4
HPV18	2	2	4	–		
HPV6/11, 16	2	–	2	1		
HPV16, 18	2	–	2	–		
Unidentifie (HPV–X)	38	3	41	15		

SCCs – squamous cell carcinoma, ADs – adenocarcinoma

tely 450 bp also from the L1 region (15). Alternatively, HPV detection by general primer PCR can be followed by HPV typing with type-specific primer pairs (TS-PCR). Although the MY09/MY11 system is slightly less sensitive than the GP5+/GP6+ system, the former is currently used more frequently for HPV screening. Both systems work well and with similar efficiency in cervical scrapes (16).

The disadvantage of all PCR-based HPV tests is the high risk of a false-positive result because of the possible cross-contamination. Therefore, these tests should be performed only in carefully controlled laboratories with experience in molecular biology.

The HC II system was developed for routine HPV detection in cervical scrapes. It is the Food and Drug Administration (FDA, USA) approved HPV test and should be used in combination with special collection kits containing sterile cotton swabs or cytobrushes and tubes with a medium suitable for storage and transport of cells and small biopsies. Instead of HPV typing, the system allows discrimination of "low-risk" and "high risk" HPV types by the use of parallel test for these risk groups. The HC II system detects anogenital HPV 18 types with a sensitivity that is slightly below that of the commonly used general-primer PCR techniques test. The test can be easily performed by laboratory staff who are not experienced in molecular biology, and the diagnosis of the HPV risk group gives enough information to the clinician in most cases. The major drawbacks of the HC II test are the impossibility of exact HPV typing and the high costs of the systems (7).

In addition, for many of the newly described HPV types, specific primers are not available. For these reasons, it is important to pursue further development of genotyping systems applicable for mass screening of cervical scrapes. Reverse hybridization line probe assay (HPV-LiPA) has been recently developed for the genotyping of a broad spectrum of HPV types in a single assay. Combined with a novel general Short Fragment Polymerase Chain Reaction (SPF) assay, amplifying a fragment of only 65 bp, this SPF-HPV-LiPA permits a highly sensitive, simultaneous detection of 16 individual HPV types in a single reaction scheme (17).

Several studies have shown the potential relevance of HPV testing in the cervical cancer screening program and a management of patients with atypical squamous cells of undetermined significance. So, taking into account the role of HPV testing in routine clinical practice, the methods of HPV identification must be useful and valuable. At present such methods are HC II and PCR. These methods were used in our study.

In conclusion, all patients (n = 92) with squamous cell carcinoma were positive for HPV infection. HPV 16 was the predominant type (52%) in the squamous cell carcinoma specimens. Among healthy volunteer women HPV was found in 26%; HPV 6/11 was the predominant type, it was detected in 8 women (31%). High-risk HPV was found in 22% of all healthy women examined.

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ŽMOGAUS PAPILOMOS VIRUSO INFEKCIJA GIMDOS KAKLELIO KARCINOMOSE

S a n t r a u k a

Šio darbo tikslas – nustatyti sveikų ir sergančių gimdos kaklelio vėžiu moterų infekuotumą žmogaus papilomos virusu (ŽPV) panaudojant DNR hibridizacijos metodą (Hybrid Capture II, HC II) bei identifikuoti jo tipus pagal polimerazės grandininę reakciją su tipams specifiniais pradmenimis (TS-PGR). Buvo tirta 100 moterų, sergančių gimdos kaklelio karcinoma: 92 moterims diagnozuota plokščialąstelinė, 8 – liaukinė, arba mišri, karcinoma. ŽPV nustatymui ir tipavimui buvo naudojama DNR hibridizacija (HC II) ir polimerazės grandininė reakcija su tipams specifiniais pradmenimis. Tarp 100 moterų, sergančių gimdos kaklelio karcinoma, 97 nustatytas ŽPV (naudojant HC II): 89 (92%) moterims identifikuoti didelės, 8 (8%) – ir didelės, ir mažos rizikos grupių virusai. Kontrolinėje grupėje (100 sveikų moterų) ŽPV infekcija konstatuota 26 moterims: 4 moterys (15%) infekuotos mažos, 14 (54%) – didelės ir 8 (31%) – ir mažos, ir didelės rizikos grupės virusais. Tiriamojoje grupėje dažniausiai buvo randamas 16 ŽPV tipas (51 moteris, 52%), kontrolinėje grupėje – 6/11 ŽPV tipas (8 moterys, 31%). Hibridizacijos metodas labiau tinka kasdieniniams tyrimams nustatant ŽPV priklausomybę didelės ar mažos rizikos grupėms. Tačiau jis yra mažiau jautrus nustatant 18 ŽPV tipą negu PGR su bendrais ar tipams specifiniais pradmenimis.