Acta Biochimica Polonica

Vol. 55 No. 4/2008, 687–692

on-line at: www.actabp.pl

Regular paper

Genotype-specific human papillomavirus detection in cervical smears★

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Received: 27 April, 2008; revised: 16 September, 2008; accepted: 03 November, 2008
available on-line: 17 November, 2008

Human papillomavirus (HPV) is widely accepted as a causative agent of cervical cancer. The distribution and prevalence of HPV types depend on geographic region and demographic factors. The aim of this study was to investigate the relationship between the presence of various HPV types and the outcome of cytological examination. Cervical smears were obtained from 125 women from southern Poland: low grade squamous intraepithelial lesions (LSIL) — 44, high grade squamous intraepithelial lesions (HSIL) — 12, cervical carcinoma — 27 and 42 women without abnormality in cytology as a control group. DNA was extracted from the smears and broad-spectrum HPV DNA amplification and genotyping was performed with the SPF 10 primer set and reverse hybridisation line probe assay (INNO-LiPA HPV Genotyping, Innogenetics). HPV DNA was detected in approximately 72% cases, more frequently in women with squamous intraepithelial lesions and cervical carcinoma than in the control group ($P<0.0005$). The most frequent type found was HPV 16 (37%), followed by HPV 51 (28%) and HPV 52 (17%). A single HPV type was detected in 51% positive cases, more frequently in cervical cancer specimens. Multiple HPV infection was dominant in women with LSIL and normal cytology. Prevalence of HPV 16 increased with the severity of cervical smear abnormality. For women HPV 16 positive, the relative risk (odds ratio) of the occurrence of HSIL and cervical cancer *versus* LSIL was 14.4 (95% CI, 3.0–69.2; $P=0.001$) and 49.4 (95% CI, 6.5–372.8; $P<0.001$), respectively. Genotyping of HPV will allow better classification of women with cervical abnormalities into different risk groups and could be useful in therapy.

Keywords: human papillomavirus, HPV genotyping, squamous intraepithelial lesions, cervical carcinoma

INTRODUCTION

Human papillomavirus (HPV) has been implicated with a high frequency in the aetiology and pathogenesis of dysplasia and carcinoma of the uterine cervix. HPV is a small (52–55 nm), nonenveloped virus with a circular, double-stranded DNA. So far, more than 100 different HPV genotypes have been identified based on differences in DNA sequence. These HPV types can be classified according to various criteria, e.g. their tissue tropism, oncogenic potential and phylogenetic classification. More than 30 types are commonly found in the genital tract. These include several HPV genotypes, such as HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 that are known as high-risk HPV, capable of causing progression to cancer in the uterine cervix, and further three genotypes (HPV 26, 53, and 66) as probable high-risk types. In contrast, other genotypes, such as HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81 have been designated as low-risk HPV and rarely, if ever, lead to cancer (Remmink *et al*., 1995; zur Hausen H., 2000; Bosch *et al*., 2002; Muñoz *et al*., 2003). Many HPV genotypes do not have an agreed-upon status.

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★Presented at the XXXV Winter School "The Structure and Function of Protein and Nucleic Acids" organized by Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, 23–27 February, 2008, Zakopane, Poland.

Abbreviations: HPV, human papillomavirus; HSIL, high grade squamous intraepithelial lesions; LSIL, low grade squamous intraepithelial lesions; SPF, short PCR fragment.

Meyer *et al*. (2001) classified HPV 53 as low-risk, and Muňoz *et al*. (2003) found discrepancies between phylogenetic and epidemiologic evidence for types 70 and 73.

Women infected with high-risk HPV types are considered to be at a higher risk for the development of cervical cancer than those who are not infected with HPV or are infected with low-risk HPV types (Remmink *et al*., 1995; Cuzik *et al*., 1995; Bosch *et al*., 2002; Muňoz *et al*., 2003; Schmidt *et al*., 2005). Papillomaviruses cannot be cultured, serological assays are useless, too. Diagnosis of HPV infection has thus to based on molecular methods, including liquid hybridisation (e.g. Hybrid Capture), Southern and dot blot hybridisation, or PCR (Manos *et al*., 1989; Low *et al*., 1990; Jacobs *et al*., 1997; Clavel *et al*., 1999; Gravitt *et al*., 2000; Finan *et al*., 2001). Various PCR-based methods have been described for the identification of HPV genotypes. Individual genotypes can be detected by type-specific PCR primer sets. However, these require the performance of multiple parallel assays for each sample, and type-specific PCR primers have not been reported for each HPV genotype. Alternatively, general PCR primer sets can be used, permitting simultaneous amplification of a broad range of HPV genotypes. The SPF-PCR system amplifies a 65 bp fragment of the L1 region which is highly conserved between HPV types (Kleter *et al*., 1998). Subsequently the amplified HPV DNA is genotyped using a reverse hybridisation line probe assay (Kleter *et al*., 1999). The present study was undertaken to determine the prevalence of HPV genotypes and assess the correlation of these findings with cytological diagnosis.

MATERIALS AND METHODS

One hundred and twenty-five cervical smears derived from women aged 20–75 (mean 39±15) years were investigated. These samples were classified as follows: normal cytology — control group (n=42), low grade squamous intraepithelial lesions — LSIL (n=44), high grade squamous intraepithelial lesions — HSIL (n=12), and cervical carcinoma (n=27), confirmed by histopathological examination using FIGO IIa/IIb stages according to the International Federation of Gynecology and Obstetrics. All cervical smears were obtained before the biopsy and were taken with a cervical brush and collected to 5 ml of phosphate-buffered saline, pH 7.2, and stored frozen at –70°C until tested.

DNA isolation. DNA was isolated using Genomic DNA Prep Plus kit (A&A Biotechnology, Gdańsk, Poland). Cell samples were lysed in a buffer with Proteinase K (1.25 mg/ml) and chaotropic salts. Addition of ethanol caused DNA to bind when the lysate was spun through a silica membrane in a microcentrifuge tube. Following washing to remove contaminants, DNA was eluted in 10 mM Tris/HCl, pH 8.5 (preheated to 75°C). The final concentration of DNA was on average 80 ng/μl. The samples were stored at +4°C according to manufacturer's instruction.

HPV DNA amplification. Broad-spectrum HPV DNA amplification was performed using a short PCR fragment (SPF_{10}) primer set (Innogenetics Inc, Gent, Belgium). The SPF_{10} primers, labelled with biotin at the 5′ end, amplified a 65 bp fragment from the L1 region of the HPV genome (Kleter *et al*., 1998).

The reaction was performed as described previously (Szostek *et al*., 2006). The final reaction volume was 50 μl and contained 10 μl of the isolated DNA, 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatine, 200 μM of each deoxynucleoside triphosphates, 15 pmol of forward and reverse primers each, and 1.5 U of Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, Ca, USA). The PCR conditions were as follows: polymerase activation 9 min at 94°C, 40 cycles of 30 s at 94°C, 45 s at 52°C, 45 s at 72°C and a final extension of 5 min at 72°C.

Each experiment was performed with separate positive (DNA from CaSki and HeLa cells) and negative (H_2O) PCR controls.

Analysis of the PCR products was performed by electrophoresis in 3% agarose gel.

β-Actin PCR analysis was performed to confirm the presence of human DNA in all specimens, according to Guzik and co-workers (1999).

HPV genotyping. Typing of the SPF_{10} amplimers was performed with reverse hybridisation using the INNO-LiPA HPV genotyping assay (Innogenetics, Gent, Belgium) which permits specific detection of 25 HPV genotypes (Kleter *et al*., 1999):
— high-risk HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70;
— low-risk HPV genotypes 6, 11, 34, 40, 42, 43, 44, 53, 54, 74.

Statistics. The frequency of infection in cervical smear abnormality groups were compared using Fisher exact test. The Kruskal-Wallis test was used to compare age in studied groups. The relative risk (odds ratio) of LSIL, HSIL and cervical cancer through HPV infection was adjusted to age and assessed using multivariate logistic regression. The analyses were carried out using the STATA 8.0 software package.

RESULTS

HPV DNA was found in 72% of the samples, more frequently in women with HSIL — 91.7% (95% CI: 61.5–99.8) and LSIL — 97.7% (95% CI: 88.0–99.9)

Table 1. HPV types in cervical smears

Cytological classification	n	HPV+ (%)	HPV types																			unidentified
			6	11	16	18	31	33	35	39	42	44	45	51	52	53	56	58	66	68	74	
Ca*	27	27 (100)		2	22	3							1	4	2				3	1		1
HSIL	12	11 (92)			7	2	1					1		3								
LSIL	44	43 (98)	3	2	4	5	5	3	1	1	4			13	11	3	2	3	3		1	7
Normal	42	9 (21)**	1	1									1	5	2	1	1		1			1
Total	125	90 (72)	4	5	33	10	6	3	1	1	4	1	2	25	15	4	3	3	7	1	1	9

*squamous cell carcinoma; ** $P<0.005$

Table 2. Single and multiple HPV types among 90 infected women

Cytological classification	Number	Number of HPV-positive women (%)				
		single	double	triple	more than triple	unidentified type
LSIL	43	15 (35)	16 (37)	3 (7)	2 (5)	7 (16)
HSIL	11	8 (73)	3 (27)	0	0	0
Ca*	27	19 (70)	3 (11)	3 (11)	1 (4)	1 (4)
Normal cytology	9	4 (44)	3 (33)	1 (9)	0	1 (9)
Total	90	46 (51)	25 (28)	7 (8)	3(3)	9 (10)

*squamous cell carcinoma

than in women with normal cytology — 21.4% (95% CI: 10.3–36.8) ($P<0.005$). The all specimens from women with cervical cancer were HPV-positive. Nineteen different HPV genotypes were detected by reverse hybridisation in this study (Table 1). Among the HPV-positive samples, the most frequent type was HPV 16 (36.7%), followed by HPV 51 (27.8%) and HPV 52 (16.7%). The incidence rates of HPV 18 infection were lower than those for HPV 16, 51, and 52. For nine cervical scrapes the HPV type could not be determined.

The occurrence of HPV 16 seems to increase with the severity of cervical smear abnormality (Fig. 1). It was not detected in the control group, and its proportion increased from 9.1% (95% CI: 2.5–21.7) in the LSIL group and 58.3% (95% CI: 27.7–84.8) in the HSIL group to 81.5% (95% CI: 61.9–93.7) in samples of cervical carcinoma. The prevalence of other HPV types was more frequent in women with LSIL (77%) than in HSIL (50%) and cervical cancer (33%) groups. These differences were statistically significant ($P<0.001$).

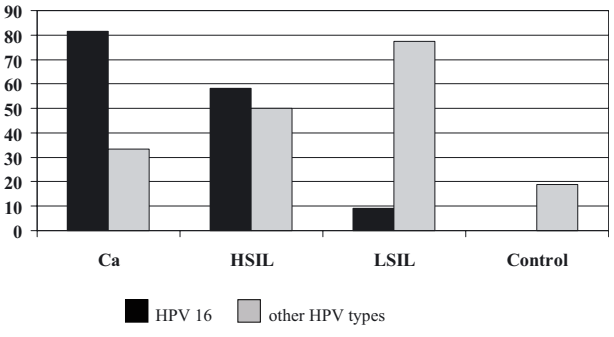

Figure 1. Frequency of HPV 16 and others HPV types in women with different cytological diagnoses

A single HPV type was detected in 51% of the positive cases (Table 2). Double infections dominated among the multiple ones. In women with a single HPV type, HPV 16 constituted 52% while in mixed infections type 51 was twice as frequent as HPV 16, 22% and 11%, respectively. A statistically significant difference was found in the numbers of detected virus types among patients with different cytological diagnoses ($P<0.0001$). In women with normal cytology or LSIL, mixed-types of HPV were significantly more frequent than in the group of women with advanced dysplastic process and cervical cancer, 48% and 26%, respectively ($P<0.05$). The relative risk of cervical carcinoma assessment by HPV type showed that HPV 16-positive cases had a 30-fold higher risk of cervical carcinoma (95% CI: 6.3–143.1; $P<0.001$). For women with HPV 16 infection, the relative risk (odds ratio) of the occurrence of HSIL and cervical cancer *versus* LSIL was, respectively, 14.4 (95% CI, 3.0–69.2; $P=0.001$), and 49.4 (95% CI, 6.5–372.8; $P<0.001$). All odds ra-

tios were adjusted to age using logistic regression analysis.

DISCUSSION

In countries that have screening programs for cervical cancer using cervical cytology significant reductions have been achieved in both the incidence and mortality from cervical cancer. In Poland, cervical cancer is the fourth most common neoplasm in women. There are 3439 new cases recorded annually, of which 53% of the patients die (Wojciechowska *et al*., 2005). HPV infections are associated with an increased risk for the development of cervical carcinoma. Therefore accurate detection of HPV infection is clinically important and requires reliable diagnostic tools.

It is known that only 1% of LSIL and about 12% of HSIL progress to invasive cervical cancer. Therefore, identification of high-risk HPV types in cervical scrapes would help to identify patients with an increased risk for development of cervical cancer. In our study the HPV prevalence was 98% in LSIL, 92% in HSIL and 100% in invasive cervical carcinoma. These data were closer to the results obtained for North America rather than for European countries (Clifford *et al*., 2003a; 2003b; 2005).

However, only 21% of women with normal cytology were found to have HPV infection, which was similar to data from southeastern Hungary (23%) obtained from comparable study groups (Nyari *et al*., 2004) and higher than data from the Czech Republic (5.3%) (Tachezy *et al*., 2003) and other Poland regions (Michalski *et al*., 2004; Bardin *et al*., 2008), but lower than the results from the Netherlands (38%) (Melchers *et al*., 1999). Studies of Perrons *et al*. (2002) showed that the prevalence of HPV in women without atypical changes in cervical epithelial cells, estimated with PCR using two different pairs of primers, was 22% for MY09/11 and 72% for SPF_{10} (Perrons *et al*., 2002). On the other hand, the frequencies of HPV infection in cytologically negative Italian women over 30 years of age were about 10% by Hybrid Capture assay and PCR, however, colposcopy revealed minor abnormalities for the majority of those women (De Francesco *et al*., 2005).

Studies carried out in other regions of Poland (Dudkiewicz *et al*., 2001; Liss *et al*., 2002; Dybikowska *et al*., 2002; Bardin *et al*., 2002) showed a lower rate of HPV infection in women with the diagnosis of LSIL (48%) and HSIL (62%) and cervical carcinoma (75%, 70%, 53%, 73.9%). These differences are probably a result of the variability of the techniques used for identification of the virus in those studies. The results of our studies confirm the data of other authors (Melchers *et al*., 1999; Perrons *et al*., 2002; Cho *et al*., 2003) that there is a direct dependence between the degree of advancement of changes within the uterine cervix and the presence of HPV infection, especially of the high-risk types. This relationship is best visible for HPV 16. In our material, the presence of this virus, in single as well as mixed infections, was noted in 81% of women with cervical carcinoma, 64% with HSIL and 9% with LSIL.

We have used SPF-10 HPV primers which had been shown to offer higher sensitivity than is available with other PCR methods using general primer sets (Kleter *et al*., 1998; Perrons *et al*., 2002). Also, the reverse hybridisation using multiple probes on line permitted simultaneous detection of 25 different HPV types. Whereas Kleter *et al*. (1999) and Quint *et al*. (2001) observed that the prevalence of multiple genotypes increased with the severity of cervical lesions and was higher in the cervical smears than in the biopsy, in the present study multiple HPV infection was more common in smears diagnosed as LSIL and normal. On the other hand, those authors agree with the fact that single types of viruses, mainly HPV 16, are present in cervical cancer. In our study, the prevailing HPV types in cervical neoplastic lesions were HPV 16 (36%), HPV 51 (28%) and HPV 52 (17%). Despite differences in the frequency of occurrence of each of the high-risk types, depending on the geographical region and the study methods used, the dominance of HPV 16 was noted in most studies (Naucler *et al*., 2004; Silins *et al*., 2004; Ronco *et al*., 2005). Similarly to De Francesco *et al*. (2005), we showed a lesser role of HPV 18 in squamous cervical cancer, which could be significant in studies relating to the composition of vaccines being prepared and their efficacy. The current vaccination strategy is based mainly on virus-like particles L1 HPV 16 and L1 HPV 18 as immunogenes (Koutsky *et al*., 2002; Harper *et al*., 2004; Frazer, 2004). In cervical cancer prophylaxis it would be usefull to include a higher number of high-risk HPV types in the vaccines. Genotyping contributes to the knowledge of the prevalence of HPV types playing a role in infections, which should then have an implication on the design of vaccines. Implementation of cervical cancer prophylaxis by HPV testing will allow better classification of patients into different risk groups and simplify the identification of patients at risk for developing this neoplastic disorder. The demonstration of persistent HPV infection, *via* multiple patient examinations and identification of the virus type, could be helpful in drawing therapeutical decisions.

Acknowledgement

This research was partially supported by grant N401219034 from the Ministry of Science and Higher Education.

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