OCCURRENCE OF HUMAN PAPILLOMAVIRUS 16 AND 18 IN SMEARS FROM THE TWO CERVIX REGIONS OF ONCO-GYNECOLOGICAL PATIENTS IN SLOVAKIA

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Summary. – We evaluated the relevance of tests for Human papillomavirus 16 and 18 (HPV-16, HPV-18) in two cervix regions (exocervical and endocervical) separately. The total of 142 cervical smears obtained from 91 women in Slovakia attending onco-gynecological outpatient care were examined for the presence of HPVs by PCR with the general primers GP5 and GP6 (GP5/6). The HPV-positive smears were examined for the presence of HPV-16 and HPV-18 and the results compared with cytological assessment. In 73 HPV-positive smears, the number of cases with detected HPV-16 was about three times higher in exocervix and about two times higher in endocervix in comparison with number of cases with detected HPV-18. In the smears considered as normal by cytology, two times higher occurrence of HPV-18 in endocervical smears was found in comparison with exocervical ones. Eight patients were double-infected with HPV-16 and HPV-18, but no patient was infected with these HPVs in both cervical regions. This finding emphasized the importance of examination of both cervical regions separately. Overlooking of the endocervical canal for the close examination by cytology and PCR might increase the failure to detect HPVs associated with adenocarcinoma.

Key words: Human papillomavirus; exocervix; endocervix; cytology; PCR

Introduction

HPV infections are associated with benign and malignant lesions of cutaneous and mucosal epithelia (zur Hausen, 1994; Munoz *et al*., 2003; Bulkmans *et al*., 2004). However, in most cases, the cervical HPV infections remain asymptomatic becoming undetectable in 1–2 years even by the most sensitive tests. While the low-risk HPV (LR HPV) types (HPV-6 and HPV-11) were usually associated with benign warts, the long-term persistence of certain high-risk HPV (HR HPV) types is strongly associated with cervical carcinogenesis (Schiffman *et al*., 2005). Both HPV types trigger growth of abnormal cells, but usually only the HR HPV infection may lead to cancer. The finding that cervical cancer could be a consequence of the infection with a few types of oncogenic HPVs led to a novel cervical screening based on HPV tests considered as the most effective method for cervical cancer control (Lörincz *et al*., 1992; Walbroomers *et al*., 1999; zur Hausen, 2000; Bosch 2002; Schiffman *et al*., 2005; Snijders *et al*., 2006).

The most predominant HR HPV in cervical cancer is HPV-16 found in 54% of cases worldwide and in 70% in Europe/North America (Munoz, *et al*., 2004). Up to now, the data for HPVs different from HPV-16 are still limited and inconsistent (Clifford, *et al*., 2003; Snijders *et al*., 2006). However, a large number of studies have established that HPV-18 is a necessary cause of cervical cancer (Walbroomers *et al*., 1999; Clifford *et al*., 2003; Munoz *et al*., 2003; Bulkmans *et al*., 2005). More recently, in addition to HPV-16 and HPV-18 other HPVs as HPV-33, HPV-45, HPV-31, HPV-58, HPV-52,

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Abbreviations: EU = European Union; GP5/6 primers = GP5 and GP6 primers; HPV = Human papillomavirus; HR HPV = high-risk HPV; LR HPV = low-risk HPV; Pap = Papanicolaou

HPV-35, and HPV-59 (in descending order of potency) have been suggested to induce invasive carcinoma (Schiffman *et al*., 2007).

A number of clinical studies demonstrated that HPV testing was more sensitive for the detection of pre-invasive stage of cervical cancer than conventional Papanicolaou (Pap) smears alone. The combination of HPV tests and cytology may achieve a negative predictive value of >97% in detecting a high-grade intraepithelial neoplasia and cervical cancer (Resnick *et al*., 1990; Clifford *et al*., 2003; Raab *et al*., 2006). Actually, the drawbacks of the current cytological examination of cervical smears, in particular the low sensitivity of the test, have enhanced the value of HPV testing. Therefore, the sensitive detection methods are required that have an important consequence for the management of patients. Current HPV testing systems are able to detect the presence of viral DNA nearly in 100% of invasive cervical cancer cases and up to 90% of its precursor stages. Additionally, the Pap smear can determine only the presence of abnormal cells, but the HPV testing has a predictive value for precancerous lesions that may later develop to cancer. HPV testing also present a significant improvement in the detection of endocervical adenocarcinoma that is difficult to detect and to prevent. The detection of HPV infection is performed by the PCR method. There are more than 40 HPV types of approximately 100 isolates infecting the anogenital tract, what have complicated the design of PCR methods for HPV detection.

The genome of HPVs consists of seven early (E1-E7) and two late (L1, L2) ORFs (zur Hausen *et al*., 1996). Beside many variable regions, most HPVs share high homology in certain regions of their genomes. These regions are used for a construction of consensus or universal primers that are able to amplify many different HPVs in one reaction. There are several consensus primers complementary to L1 and E6/E7 regions of HPV genome (Manos *et al*., 1989; Snijders *et al*., 1990; Yoshikawa *et al*., 1990; Resnick *et al*., 1990). During the integration of viral DNA into the host DNA, parts of the L1 region could be deleted (Schwarz *et al*., 1985; Choo *et al*., 1988) and consequently consensus primers that are not located in the L1 region are preferred. In comparison with E6/E7 region primers, L1-PCR has a broader spectrum of HPV detection. The general primers GP5/6 are complementary to the part of the L1 regions for at least six genital HPVs (HPV-6, 11, 16, 18, 31, 33) (Snijders *et al*., 1990).

In this study, we detected presence of HPV in exocervical and endocervical smears. Usually the smears collected from both regions of cervical canal were blended and then used for the cytological examination as well as for the PCR detection. In our experiments, we collected the smears separately and compared the results of HPV detection and cytology. A collection of 142 smears obtained from the women diagnosed with onco-gynecological ailments were examined by HPV PCR with general GP5/6 primers. Next, the HPV-positive samples were tested for the presence of HPV-16 and HPV-18 and the results were compared to the cytological results. This study represents the first report about the incidence of HPV-16 and HPV-18 in smears collected from both cervix regions separately.

Materials and Methods

Patients. The female patients from western Slovakia were recruited from gynecological practices in the year 1995 to 2003 because of abnormal cytological results. The total of 142 cervical smears were obtained from 91 women. The smears were collected from exocervix (51 cases) and/or endocervix (91 cases). Hence, 51 patients were simultaneously tested in both cervical regions. The average age of women was 38 years (from 24 to 66 years). The smears for cytological as well as PCR examination were collected with a cotton swab (van den Brule *et al*., 1992). A pellet of exfoliated cells was resuspended in 200 µl of distilled water, heated at 95ºC for 10 mins, quickly chilled, centrifuged, and supernatant containing crude DNA was used in the PCR procedure.

Cytology. Examined women were subjected to the cytological examination of cervico-vaginal Pap smears interpreted for the presence of cervical intraepithelial neoplasia according to the conventional methods (Syrjänen, 2000). Classification of Pap smears: Pap I, II – no pathologic change, Pap III – Atypical Squamous Cells of Undetermined Significance (ASCUS), Pap IIIa – mild dysplasia, Pap IIIb – moderate or severe dysplasia, Pap IV – Atypical Glandular Cells of Undetermined Significance (AGCUS).

HPVs PCR. To optimize the procedure of PCR with GP5/6 primers using crude cellular DNA for HPV-6, 11, 16, 18, 31, and 33 detection, the original protocol was followed with slight modifications (van den Brule *et al*., 1990). Briefly, PCR mixture containing 3.5 mmol/l $MgCl_2$, 50 pmol/l of each of GP5/6 primers, and about 1 µg of DNA from cytological smear was incubated for 5 mins at 95°C for DNA denaturation and then subjected to 45 cycles of amplification (each cycle consisted of a denaturation step at 95°C for 1 min, primer annealing step at 40°C for 2 mins, and a chain elongation step at 72°C for 1.5 min). To control internally the quality of the DNA, the 268 bp sequence of ß-globin gene was amplified using PC04 and GH20 primers (Bell *et al*., 1993). The DNA isolated from cervical carcinoma cell lines CaSki (containing from 60 to 600 copies of HPV-16) or HeLa (containing from 10 to 50 copies of HPV-18) was used as a positive control (Meisner, 1999). Finally, 25–40 µl of the amplification product was analyzed by 1.5 or 2% agarose gel electrophoresis.

Table 1. Examination of exocervical and endocervical smears for HPVs, HPV-16, and HPV-18

No. of smears (region)	No. (%) of positive smears				
	HPVs[1]	HPV-16[2]	HPV-18[2]	HPV-16 and HPV-18[2]	Total
51 (exocervix)	25 (49.0)	9 (36.0)	3 (12.0)	5 (20.0)	17 (68.0)
91 (endocervix)	48 (52.7)	17 (35.4)	7 (14.6)	6 (12.5)	30 (62.5)

[1]HPVs PCR using primers GP5/6.
[2]Type-specific HPV-16, 18 PCR and Southern blot analysis.

HPV-16 and HPV-18 PCR. The PCR with specific primers (TS16 and TS18) was performed as described above, except that 1.5 mmol/l $MgCl_2$, 25 pmol/l of each type specific primer set, and an annealing temperature of 55°C was used. Type specific PCR products were analyzed as described above.

Southern blot analysis. Hybridization of blotted PCR products was performed under high-stringency conditions with either HPV-16- or HPV-18-specific [γ-^{32}P] dCTP labeled oligonucleotides (van den Brule *et al*., 1990).

Results and Discussion

HPVs PCR

The specimens collected from 91 patients (51 exocervical and 91 endocervical) were tested by PCR with general primers GP5/6 detecting HPV-6, 11, 16, 18, 31, and 33. The DNA samples were ß-globin-positive and suitable for further analysis. The presence of at least one of HPVs e.g. HPV-6, 11, 16, 18, 31, and 33 was detected in 52.7% of endocervical and 49% of exocervical smears (Table 1).

HPV-16 PCR and HPV-18 PCR

In 73 samples that were PCR-positive with GP5/6 primers, the presence of two HR HPVs (HPV-16 or HPV-18) was tested by PCRs with type-specific primers and Southern blot analysis. There were 17 samples from exocervix and 30 samples from endocervix positive for either HPV-16 or HPV-18 or for both of them (Table 1). The prevalence of HPV-16 and/or HPV-18 infection in exocervical smears was 68% and in endocervical 62.5%. The frequency of HPV-16 infection in comparison with that of HPV-18 was about three times higher in exocervix and about two times higher in endocervix. Furthermore, the results showed that exocervix was more frequently infected with both HR HPVs than endocervix. On the other hand, approximately in a half of exocervical as well as endocervical smears was detected only one of the two HPVs examined. In all cervical smears, we found relatively high prevalence of HPV-16 and/or HPV-18 reaching about 33% (47 cases out of 142 smears) in both exocervix and endocervix. The rate of HPV-16 infection (either alone or in co-infection with HPV-18) in all smears was near 26%.

Comparison of PCR results with cytological examination

Results of HPV PCR obtained with GP5/6 primers and type-specific PCR/Southern blot analysis in 91 endocervical and 51 exocervical smears were compared with cytological examination. The 32 endocervical and 22 exocervical smears were examined by cytology as normal (Pap I or Pap II) and at the same time detected by PCR with GP5/6 primers as HPV-positive (Table 2). In this set of specimens, 20 endocervical and 14 exocervical samples were positive either for HPV-16 or HPV-18 or for both of them. The exocervical smears were more frequently double-infected (5 samples from 22 tested) than endocervical (4 samples from 32 tested). In the group of HPV-positive smears assessed by cytological examination as mild dysplasia (Pap IIIa), HPV-16 and/or HPV-18 prevalence was higher than in the smears assessed by cytological examination as normal. Furthermore, the presence of HPVs other than HPV-16 or HPV-18 was confirmed in abnormal endocervical smears, but not in exocervical ones. However, it is worth noting that this study examined a selected group of patients who were recruited from gynecological practices because of repeatedly abnormal cytological results and later were treated in onco-gynecological outpatients care. Hence, some data obtained in this study were not applicable for a healthy population. The majority of smears were assessed as normal by cytology (Pap I and Pap II). In spite of this, 88% of exocervical and 66% of endocervical smears were HPV DNA-positive (Table 2). In smears tested normal in cytology, two times higher prevalence of HPV-18 in was found in the endocervical than exocervical smears. It was noteworthy that the number of endocervical smears examined by cytology as normal was about two times higher HPV-16-positive (or HPV-16 and HPV-18-positive), than the exocervical smears. Recently, it was shown that both HPV-16/18 infections in women with normal cytology were associated with an increased risk for

Table 2. Examination of exocervical and endocervical smears by PCR, Southern blot analysis, and cytological assessment

Cytological assessment	Pap I, II	Pap IIIa	Pap IIIb	Pap IV
Endocervical smears				
HPVs-positive[1]	32/48 (66.6)	10/48 (20.8)	4/48 (8.3)	2/48 (4.1)
HPV-16-positive[2]	10 (31.2)	6 (60.0)	1 (25.0)	0
HPV-18-positive[2]	6 (18.2)	1 (10.0)	0	0
(HPV-16 and HPV-18)-positive[2]	4 (12.5)	0	1 (25.0)	1 (50.0)
Total	20/32 (62.5)	7/10 (70.0)	2/4 (50.0)	1/2 (50.0)
Other HPVs-positive	12/32 (37.5)	3/10 (30.0)	2/4 (50.0)	1/2 (50.0)
Exocervical smears				
HPVs-positive[1]	22/25 (88.0)	1/25 (4.0)	2/25 (8.0)	0
HPV-16-positive[2]	7 (31.8)	1 (100.0)	1 (50.0)	0
HPV-18-positive[2]	2 (9.1)	0	1 (50.0)	0
(HPV-16 and HPV-18)-positive[2]	5 (22.7)	0	0	0
Total	14/22 (63.6)	1/1 (100.0)	2/2 (100.0)	0
Other HPVs-positive	8/22 (36.3)	0	0	0

[1]HPVs PCR.
[2]Type-specific HPV-16, 18 PCR and Southern blot analysis.

high-grade lesions and cervical cancer (Castle *et al*., 2005; Khan *et al*., 2005). Recently, the prevalence of HPV-18 infections preferentially increased in the cervical adenocarcinoma (Vizcaino *et al*., 1998; Woodman *et al*., 2003). Our results reflected also the lesser value of cytological assessment for HR HPV infection in comparison with the HPV-16 and HPV-18 detection by PCR. Recently, a novel method has been suggested for improvement of the Pap test quality and for the decrease of medical errors (Raab *et al*., 2006). A new development in cytology, such as liquid-based techniques and automated reading seem to overcome effectively some of conventional Pap test limitations and have the potential to improve sensitivity and specificity of cytology in diagnosis and screening for cervical pre-cancer and cancer cases (Davey *et al*., 2006, 2007). Nevertheless, even the high sensitive test with combination of cytology and HR HPV typing cannot predict the biological potential of prevalent cervical pre-cancer case towards a progression or regression. This prediction can be assessed only by using an adequate biomarker of neoplastic transformation, e.g. chromosomal aneuploidy in combination with the morphological and HPV tests (Bollmann *et al*., 2003, 2005).

Results of HPV PCR detection in paired exocervical and endocervical smears

In the group of 51 patients having paired specimens (from both cervical regions), positive results were obtained for 28 patients in PCR with GP5/6 primers. For these patients, the presence of HPV was detected either in both cervical regions (19 patients) or in one region only (4 patients in exocervix and in 5 patients in endocervix). The presence of HPV in both cervical regions was negative in 23 cases (45.1%). Next, the HPV-positive patients (28 cases) were typed for the presence HPV-16, 18 (Table 3). Sixteen patients (57.2%) were HPV-16-positive and 10 patients (35.7%) were HPV-18

Table 3. Presence of HPV-16 and HPV-18 in paired exocervical and endocervical smears detected by PCR

Exocervical smears		Endocervical smears					
		HPV-16		HPV-18		HPV-16 and HPV-18	
		Positive	Negative	Positive	Negative	Positive	Negative
HPV-16	Positive	4	0	1	0	0	3
	Negative	0		0		0	
HPV-18	Positive	0	0	0	0	1	2
	Negative	0		0		0	
HPV-16 and	Positive	2	0	3	0	0	0
HPV-18	Negative	1		0		1	10

positive at least in one cervical region. To be exact, 12 patients (42.8%) were negative at both cervical regions for HPV-16 and 18 patients (64.3%) for HPV-18, respectively. Independently on the cervical region tested, 8 patients (28.6%) were found double-infected with HPV-16 and HPV-18. The fact that no patient tested in both cervical regions was infected with both HPV-16, 18 supported the necessity to examine both cervical regions separately. Together, 17 patients had HPV-16 or HPV-18 negative results at least in one cervical region. However, these patients were positive in HPV PCR with GP5/6 primers hence were infected with other HPVs.

It is of interest that the presence of HPV-16 only (without co-infecting HPV-18) was confirmed in both cervical regions just for 4 patients. That means that at least for several patients, detection of HPV-16 should be successful only in the case of examination of both cervical regions. HPV-16, but not HPV-18 was detected as infecting virus in both cervical regions at the same time. These results suggested that HPV-18 detected in the endocervix or in exocervix is apparently not the only infecting HR HPV present in the cervix.

In this study, the HPV detection results performed on the smears collected from two regions of cervix in the small group of onco-gynecological patients confirmed relatively high incidence of HPV-16 that is also primarily detected in cervical carcinoma worldwide (Munoz *et al*., 2004; Bulkmans *et al*., 2005; Schiffman *et al*., 2005, 2007). The observed higher incidence of HPV-16 and HPV-18 in endocervical and exocervical smears as well as recently reported increasing occurence of adenocarcinoma in some countries underlined the necessity to devote more attention to the identical examination of both exocervical and endocervical regions (Bulkmans *et al*., 2004; Bulk *et al*., 2005).

Carcinoma of the cervix uteri is the second most common cancer occurring in women worldwide. In former European Union (EU), the cervical cancer was estimated in 3% of cancers in women and was the tenth most common cause of cancer-related deaths in women in the year 1998 (Ferlay *et al*., 1999). In the countries of eastern and central Europe, a substantial rise in female mortality caused by cervical cancer was recently documented (Levi *et al*., 2004). This is the first report about HPV-16 and HPV-18 prevalence in smears of the two regions of cervix in Slovakian women and may contribute to the monitoring of the cervix cancer epidemiology in this country.

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References

Bell DA, Taylor JA, Paulson DF, Robertson CN, Mohler JL, Lucier GW (1993): Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen- metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer. *J. Natl. Cancer Inst*. **85**, 1159–1164.

Bollmann R, Mehes G, Torka R, Speich N, Schmitt C, Bollmann M (2003): Determination of features indicating progression in atypical squamous cells with undetermined significance: human papillomavirus typing and DNA ploidy analysis from liquid-based cytologic samples. *Cancer* **99**, 113–117.

Bollmann R, Bankfalvi A, Griefingholt H,Trosic A, Speich N, Schmitt C, Bollmann M (2005): Validity of combined cytology and human papillomavirus (HPV) genotyping with adjuvant DNA-cytometry in routine cervical screening: results from 31031 women from the Bonn-region in West Germany. *Oncol. Report* **13**, 915–922.

Bosch FX, Lörincz A, Munoz N, Meijer CJ, Shah KV (2002): The causal relation between human papillomavirus and cervical cancer. *J. Clin. Pathol*. **55**, 244–265.

Bulk S, Visser O, Rozendaal L, Verheijen RH, Meijer CJ (2005): Cervical cancer in the Netherlands 1989–1998: Decrease of squamous cell carcinoma in older women, increase of adenocarcinoma in younger women. *Int. J. Cancer* **1**, 1005–1009.

Bulkmans NW, Rozendaal L, Snijders PJ, Voorhorst FJ, Boeke AJ, Zandwijken GR, van Kemenade FJ, Verheijen RH, v Groningen K, Boon ME, Keuning HJ, van Ballegooijen M, van den Brule AJ, Meijer CJ (2004): POBASCAM, a population-based randomized controlled trial for implementation of high-risk HPV testing in cervical screening: design, methods and baseline data of 44,102 women. *Int. J. Cancer* **110**, 94–101.

Bulkmans NW, Bleeker MC, Berkhof J, Voorhorst FJ, Snijders PJ, Meijer CJ (2005): Prevalence of types 16 and 33 is increased in high-risk human papillomavirus positive women with cervical intraepithelial neoplasia grade 2 or worse. *Int. J. Cancer* **117**, 177–181.

Castle PE, Solomon D, Schiffman M, Wheeler CM (2005): Human papillomavirus type 16 infections and 2-year absolute risk of cervical precancer in women with equivocal or mild cytologic abnormalities. *J. Natl. Cancer Inst.* **97**, 1066–1071.

Choo KB, Lee HH, Pan CC, Wu SM, Liew LN, Cheung WF, Han SH (1988): Sequence duplication and internal deletion in the integrated human papillomavirus type 16 genome cloned from a cervical carcinoma. *J. Virol.* **62**, 1659–1666.

Clifford GM, Smith JS, Aguado T, Franceschi S (2003): Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. *Br. J. Cancer* **89**, 101–105.

Davey E, Barratt A, Irwing L, Chan SF, Macaskill P, Mannes P, Saville AM (2006): Effect of study design and quality

on unsatisfactory rates, cytology classifications, and accuracy in liquid-based versus conventional cervical cytology: a systematic review. *Lancet* **367**, 122–132.

Davey E, d'Assuncao J, Irwig L, Macaskill P, Chan SF, Richards A, Farnsworth A (2007): Accuracy of reading liquid based cytology slides using the ThinPrep Imager compared with conventional cytology: prospective study. *BMJ* **335**, 31–38.

Ferlay J, Bray F, Sankila R, Parkin DM (1999): EUCAN: Cancer Incidence, Mortality and Prevalence in the European Union 1998. version 5.0: IARC CancerBase No. 5. Lyon, IARC Press.

Khan MJ, Castle PE, Lorincz AT, Wacholder S, Sherman M, Scott DR, Rush BB, Glass AG, Schiffman M (2005): The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J. Natl. Cancer Inst.* **97**, 1072–1079.

Krane JF, Granter SR, Trask CE, Hogan CL, Lee KR (2001): Papanicolaou smear sensitivity for the detection of adenocarcinoma of the cervix: a study of 49 cases. *Cancer* **93**, 8–15.

Levi F, Lucchini F, Negri E, La Vecchia C (2004): Trends in mortality from major cancers in the European Union, including acceding countries, in 2004. *Cancer* **101**, 2843–2850.

Lörincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ (1992): Human papillomavirus infection of the cervix: Relative risk association of 15 common anogenital types. *Obstet. Gynecol.* **79**, 328–337.

Manos MM, Ting Y, Wright DK, Lewis AI, Broker TR, Wolinsky SM (1989): The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells* **7**, 209–214.

Meisner JD (1999): Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines. *J. Gen. Virol.* **80**,1725–1733.

Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ (2003): Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N. Engl. J. Med.* **348**, 518–527.

Munoz N, Bosch FX, Castellsague X, Diaz M, de Sanjose S, Hammouda D, Shah KV, Meijer CJ (2004): Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int. J. Cancer* **111**, 278–285.

Raab SS, Andrew-Jaja C, Condel JL, Dabbs DJ (2006): Improving Papanicolaou test quality and reducing medical errors by using Toyota production system methods. *Am. J. Obstet. Gynecol.* **194**, 57–64.

Resnick RM, Cornelissen MTE, Wright DK, Eichinger GH, Fox HS, ter Schegget J, Manos MM (1990): Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J. Natl. Cancer Inst.* **82**, 1477–1484.

Schiffman M, Herrero R, DeSallec R, Hildesheim A, Wacholder S, Rodriguez AC, Bratti MC, Sherman ME, Morales J, Guillen D, Alfaro M, Hutchinson M, Wright TC, Solomon D, Chen Z, Schussler J, Castle PE, Burk RD (2005): The carcinogenicity of human papillomavirus types reflects viral evolution. *Virology* **337**, 76– 84.

Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S (2007): Human papillomavirus and cervical cancer. *Lancet* **370**, 890–907.

Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, zur Hausen H (1985): Structure and transcription of human papillomavirus sequence in cervical carcinoma cells. *Nature* **314**, 111–114.

Snijders PJF, van den Brule AJF, Schrijnemakers HJF, Snow G, Meijer CJLM, Walboomers JMM (1990): The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. *J. Gen. Virol.* **71**, 173–181.

Snijders PJ, Hogewoning CJ, Hesselink AT, Berkhof J, Voorhorst FJ, Bleeker MC, Meijer CJ (2006): Determination of viral load thresholds in cervical scrapings to rule out CIN 3 in HPV16, 18, 31 and 33-positive women with normal cytology. *Int. J. Cancer* **119**, 1102–1107.

Syrjänen KJ (2000): Management of abnormal PAP smears (MAPS): Implications of terminology used in cytopathology. *J. Lower Genital Tract Dis.* **4**, 217–223.

van den Brule AJC, Meijer CJLM, Bakels V, Kenemans P, Walboomers JMM (1990): Rapid detection of human papillomavirus in cervical scrapes by combined general primer-mediated and type-specific polymerase chain reaction. *J. Clin. Microbiol.* **28**, 2739–2743.

van den Brule AJC, Snijders PJ, Raaphorst PM, Schrijnemakers HF, Delius H, Gissmann L, Meijer CJ, Walboomers JMJ (1992): General primer polymerase chain reaction in combination with sequence analysis for identification of potentially novel human papillomavirus genotypes in cervical lesions. *J. Clin. Microbiol.* **30**, 1716–1721.

Vizcaino AP, Moreno V, Bosch FX, Munoz N, Barros-Dios XM, Parkin DM (1998): International trends in the incidence of cervical cancer. I. Adenocarcinoma and adenosquamous cell carcinomas. *Int. J. Cancer* **9**, 536–545.

Walbroomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PF, Peto J, Meijer CJ, Munoz N (1999): Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189**,12–19.

Woodman CB, Collins S, Rollason TP, Winter H, Bailey A, Yates M, Young LS (2003): Human papillomavirus type 18 and rapidly progressing cervical intraepithelial neoplasia. *Lancet* **361**, 40–43.

Yoshikawa H, Kawana T, Kitagawa K, Mizuno M, Yoshikura H, Iwamoto A (1990): Amplification and typing of multiple cervical cancer-associated human papillomavirus DNAs using a single pair of primers. *Int. J. Cancer* **45**, 990–999.

zur Hausen H (1994): Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. *Curr. Topics Microbial. Immunol*. **186**, 131–155.

zur Hausen H (1996): Papillomavirus infections – a major cause of human cancers. *Biochim. Biophys. Acta* **1288**, F55–F78.

zur Hausen H (2000): Papillomaviruses causing cancer: evasion from host cell control in early events in carcinogenesis. *J. Natl. Cancer Inst*. **92**, 690–698.