Impact of sampling origin on molecular detection of high-risk human papillomavirus and oncogene expression

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Summary

Purpose of investigation: The recognition of high-risk human papillomavirus (HR-HPV) as an etiological agent of cervical cancer has increased the importance of testing for HPV, and this might contribute to better risk stratification. *Methods:* Eighty-eight randomly selected cervical cancer specimens including biopsies and their respective smears were used in this study. Control scrapings were obtained from ten healthy women. The presence of HPV16 and HPV18 was investigated using the technique of polymerase chain reaction (PCR) with the specific primers for the L1 region, while mRNA expression of HPV16 E6-E7 was evaluated by a reverse transcription PCR method (RT-PCR). *Results:* The positivity for the viral genotype was influenced by the quantity of amplified DNA used. In tumor biopsies the higher positivity for HPV16 (54.5%) and HPV18 (15.9%) was obtained using 687.4 ng of DNA. At smears level solely 31.8% of HPV16 was detected using an average DNA quantity of about 157.2 ng. The revelation of HPV types depends on clinicopathologic data; HPV16 was detected more in advanced stages of squamous carcinoma (SC) samples (20% Stage I, 62% Stage II and 80% Stage III), while HPV18 and double infection were found exclusively at advanced stages of SC and in adenocarcinoma (AC), respectively (60%, 40% Stage III SC and 80%, 20% Stage II A and C). The prevalence of HPV16 E6-E7 transcripts was evaluated at tumor biopsy with frequencies of 50%. *Conclusion*: Our data provide prospective evidence that HPV16/18L1 revelation at biopsy toward pathological types is efficient and correlates well with oncogenic transcript findings. Subtle changes in viral oncogene dynamics highlight the presence of other regulating proteins serving as additional biomarkers.

Key words: Biopsies; Cervical cancer; DNA load; E6-E7 oncogenes; Human papillomavirus; L1 gene; Smears.

Introduction

Cervical cancer is a major problem in women's health. It is the second most common cancer in the world and the leading cause of cancer death among women in developing countries. Worldwide, an estimated 500,000 new cases occur and 250,000 women die annually from this tumor [1]. In Tunisia, the standardized cervical cancer incidence is 5.91 per 100,000 women yearly [2]. Clinical, molecular and epidemiological investigations have identified HPV as the major cause of cervical cancer and cervical dysplasia [3, 4]. This virus is sexually transmitted and the male is the carrier. More than 100 HPV genotypes have been described and 20 of them have been associated with cervical cancer [5]. Among the high-risk types, HPV16 and HPV18 are the most closely associated with cervical carcinoma [6, 7]. HPV18 is particularly interesting, since it is reported to be mainly associated with adenocarcinoma (AC), while HPV16 is more frequent in squamous carcinoma (SC) [8, 9]. Cytological examination of cervical smears is the most widely applied screening method for cervical cancer and its precursors. However, success of the smear test is limited with respect to sensitivity. Histological testing will be required in order to evaluate HPV detection. Because of the strong association between HPV infection and cervical cancer, detection of HPV DNA in cervical samples may be an available option to identify women at risk of developing cancer [10]. Numerous molecular techniques have been used, essentially the polymerase chain reaction (PCR) which is widely used for routine clinical practice. For comparison and additional evaluation, the detection of E6 and E7 transcripts of HR-HPV could serve as a better risk evaluation factor than DNA detection for the development of a high-grade squamous intraepithelial lesion and the progression to cervical carcinoma [11]. Specific HR-HPV transcripts E6 and E7 have been shown to act as oncogenes [12]; E6 and E7 proteins inactivate the tumor suppressor p53 and retinoblastoma (Rb) respectively and induce the breakdown of cell cycle regulation. Hence, HR-HPV infected cells develop genomic instability which can lead to the progression of cancer [13, 14]. Due to methodological reasons in large studies, we investigated in this work whether the origin of sampling affects viral load and molecular presence of the main high-risk viruses. Furthermore, we studied the screening impact on AC and SC of the cervix in different tumor stages. Ultimately the HPV16 E6-E7 transcripts were evaluated to correlate with HPV16 DNA findings.

Materials and Methods

Clinical samples

The study was retrospectively performed on 88 cervical cancer specimens including biopsies and respective smears. From each patient, cervical scraping was taken using an Ayre spatula harvested at once with biopsy then collected in 1 ml PBS (phosphate-buffered saline, pH 7.4). These samples were

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Table 1. — *Polymerase chain reaction and reverse transcription primers, product length and programs.*

Primers	Designation	Product length (bp)	PCR program
BHPV 16 L1 sense	5'-GCAAGCAACAGTTACTGCGACGT-3'	301	94°C 1', 58°C 1',72°C 1'; X 40
HPV16 L1 anti-sense	5'-GCAACAAGACATACATCGACCGG-3'		
HPV18 L1 sense	5'-AAGGATGCTGCACCGGCTGA-3'	217	94°C 1', 66°C 1',72°C 1'; X 40
HPV18 L1 anti-sense	5'-CACGCACACGCTTGGCAGGT-3'		
β-globin sense	5'-CAACTTCATCCACGTTCACC-3'	268	95°C 1', 55°C 1',72°C 1'; X 40
β-globin anti-sense	5'-GAAGAGCCAAGGACAGGTAC-3'		
HPV 16 E6 sense	5'-TTACCACAGTTATGCACAGA-3'	300	94°C 30s, 50°C 30s, 72°C 1'; X 30
HPV16 E6 anti-sense	5'-ACAGTGGCTTTTGACAGTTA-3'		
HPV16 E7 sense	5'-AGAAACCCAGCTGTAATCAT-3'	300	94°C 30s, 50°C 30s, 72°C 1'; X 30
HPV16 E7 anti-sense	5'-TTATGGTTTCTGAGAACAGA-3'		
β-actin sense	5'-AGCCATGTACGTTGCTATCC-3'	500	94°C 30s, 50°C 30s, 72°C 1'; X 30
β-actin anti-sense	5'-TTGGCGTACAGGTCTTTGC-3'		

provided from 44 Tunisian patients with cervical cancer who were being treated at the Department of Radiotherapy in Salah Azaiez Institute (Tunis, Tunisia). The medium age of these patients was 57 years with a range of 38-76 years. Histological classification was done according to the World Health Organization (WHO) criteria and consisted of 39 SCs and five ACs. Clinically, tumors were staged according to the guidelines of the International Federation of Obstetrics and Gynaecology (FIGO) classification of tumors and all patients had Stage I to III of the disease. Control specimens were provided from ten healthy women attending the Obstetrics and Gynecology Department of La Rabta Hospital (Tunis, Tunisia) without any previous history of cervical malignancy. Control samples consisted of normal cervical scrapes which tested negative for cancer by a smear test. They were derived from married women aged between 20 and 53 years (median age: 36 years). All specimens were collected in saline solution and stored frozen at –20°C until processed.

Nucleic acid isolation

Viral DNA was extracted from cervical specimens using a commercially available kit (QIAamp DNA Mini Kit, Qiagen), according to the manufacturer's instructions. Briefly, after enzymatic digestion by protease K (56°C, 2 hr), the lysates were loaded onto the Qiamp columns. After two washes, total DNA was eluted in 100 µl of elution buffer and stored frozen at -20°C for subsequent virological examination. RNA extraction was carried out using RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations. The material was divided into smaller pieces while it was kept on dry ice and transferred to 600 µl of lysis buffer, followed by 30 sec of homogenization with disposable pestles. The pellets were vacuum dried for 15 sec and the RNA dissolved in 30 µl RNase free water. The integrity, purity and the concentration of the target nucleic acids was assessed by spectrophotometry.

PCR assay

The presence of HR-HPV in cervical cells was detected by the PCR method using specific primers which were designed to amplify the late L1 conserved region of HPV16 and HPV18 (synthesized by GENECUST, France). PCR was performed in a final reaction mixture volume of 100 µl containing 15 µl of DNA sample, 1.5 µl of each primer, 1.5 mM Mg Cl2, 50 mM KCl, 10 mM Tris HCl, 200 µM of each dNTP (deoxynucleoside triphosphate) and 2.5 U of taq polymerase (Fermentas). DNA was amplified using a PCR thermocycler (Applied Biosystem). Primers and thermal cycler programs for identification of each virus are listed in Table 1 [15]. Each PCR experiment was performed with positive (HPV plasmids) and negative (water) controls. The quality of DNA obtained was controlled by amplification with primers detecting the housekeeping gene β-Globin. Finally, amplified DNA obtained by PCR from each sample was examined by electrophoresis on 1.5% agarose gels (Sigma) stained with ethiduim bromide, visualized under ultraviolet light and photographed. DNA bands with the appropriate size were identified by comparison with a DNA ladder of known molecular weight (promega).

Reverse transcription PCR for detection of HPV16 E6 and E7 transcripts

HPV16 positive samples were further subjected to amplification of E6 and E7 transcripts. To control RNA integrity, PCR reactions using β-actin specific primers were performed as described previously [16] (Table 1). We processed 1 µg total RNA that was reverse-transcribed using the one-step RT-PCR Kit (Qiagen) in a 50 µl reaction containing 1X Qiagen one step RT-PCR buffer, 400 µM of each dNTP, 0.6 µM random primers and 2 µl Qiagen one-step RT-PCR enzyme. The reaction was allowed to proceed for 30 min at 50°C for reverse transcription and 15 min at 95°C for initial PCR activation step. Cycling programs performed are illustrated in Table 1. The amplified products were electrophoresed in 1.5% agarose gel, marked by a 100-pb DNA ladder (Gene Ruler, Fermentas), stained with ethiduim bromide, and visualized under UV light.

Statistics

Statistical analyses were performed using Statistica, version 6.0, for windows. The relationships between the different variables were assessed using Fisher's exact test. Differences in detection of HPV16 DNA L1 and mRNA E6-E7 in biopsies were evaluated using the two-tailed McNemar's test. When the *p* value less than 0.05 the difference was considered statistically significant.

Results

Prevalence of HPV genotypes in cervical cancer cells

The amount of DNA quantified by ultraviolet spectrophotometer varied widely, and the mean value was 56.2 µg. Variations in DNA quantities reflects the difference on number cells in each sample. The quantity of PCR assay affects viral detection. Thus, in tumor biopsies

Table 2. — *Quantitative evaluation of DNA according to cellular rates and prevalence of the HPV types on the level of tumor biopsies and smears.*

Parameters	Whole DNA recuperated (µg)	Cells number X 10^6	DNA used for PCR (ng) in positive	HPV prevalence
Biopsies				
HPV16				(24/44) 54.5%
HPV18	91.6 ± 24.4	15.2 ± 4	687.4 ± 182.9	(7/44) 15.9%
HPV16+HPV18				(3/44) 6.8%
Smears				
HPV16				(14/44) 31.8%
HPV18	20.9 ± 22.9	3.2 ± 4	157.2 ± 178.4	0%

Data is presented as mean ± S.D.
P: value for the differences between HPV DNA types distribution in smears and biopsies. (*P* Fisher's exact test).

Table 3. — *Prevalence of HPV DNA types according to the clinicopathologic data.*

Items	Total no. of samples	HPV 16 positive n (%)	HPV 18 positive n (%)	*p* value
Pathologic type				
SC	39	23 (58.9%)	3 (7.6%)	0.005*
AC	5	1 (20%)	4 (80%)	
Stage				
Early (I, II)	39	20 (51.2%)	4 (80%)	
Late (III)	5	4 (10.2%)	3 (60%)	0.17

SC = squamous carcinoma; AC = adenocarcinoma.
(P: Fisher's exact test).

Table 4. — *Comparison between L1 DNA and E6-E7 RNA for HPV16 detection.*

	No. of specimens	HPV 16 Status HPV positive	HPV negative
PCR L1 gene	44	24 (54.5%)	20 (45.4)
RT-PCR E6-E7 transcripts	44	22 (50%)	22 (50%)

(*P*: two-tailed McNemar's test).

the higher positivity for HPV16 (54.5%) and HPV18 (15.9%) was obtained using 687.4 ng of DNA. Among smear specimens only HPV16 (31.8%) was found with a lower DNA amount (157.2 ng). The relationship between the detectability of HPV types in smears and biopsies is approximated to a statistically significant level ($p = 0.05$) (Table 2). In the cancer specimens, three cases were positive for HPV16 and HPV18 (6.8%). In all scraping samples of the control group, HPV DNA was not detected.

HR-HPV infection was determined by the PCR method. Figure 1 demonstrates the results of agarose gel electrophoresis of PCR products. The β-globin gene was used as an internal control to ensure the quality of DNA in all samples. The desired strips were clear and conspicuous (Figure 1A).

Using L1 primer, HPV DNA was detected according to sampling origin. In Figure 1 B and C, HPV16 and HPV18 positive cases are shown as examples.

Correlation between HPV status and clinicopathologic data

The prevalence of HPV types according to the histological data and stages of cervical carcinoma is listed in

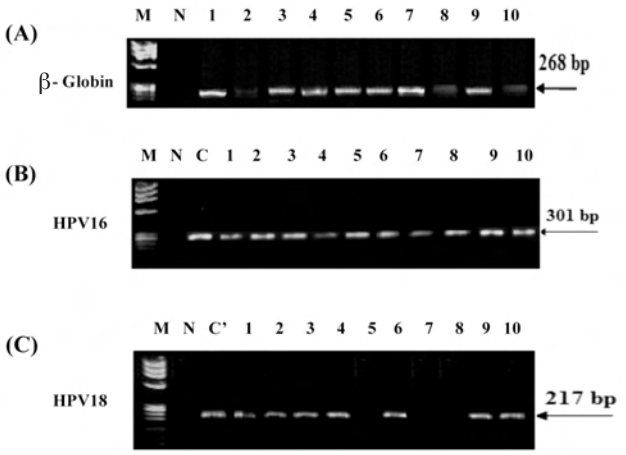

Figure 1. — Gel electrophoresis of PCR products for HPVL1 typing. (A) β-globin as internal control. (B) Positive samples for HPV-16. Lane 1-5: HPV16 in biopsies; lane 6-10: HPV16 in smears, (C) HPV-18 positive in biopsies, M; indicates size markers of *HaeIII*-digested phage Φ X174 DNA, N; negative control (water), C; positive control (HPV16 plasmid), C'; (Cervical carcinoma previously proven to contain HPV18).

Table 3. There was a significant correlation between the frequency detection of HPV genotypes according to histological types ($p = 0.005$, Table 3). Differences in frequency between HPV16 and HPV18 were not statistically significant with regard to the various stages of cervical carcinoma (Table 3). Meanwhile these data revealed that there was a tendency of increased HR-HPV positivity when the lesion was more severe. HPV16 L1 amplification indicated that 20% (1/5), 62% (18/29) and 80% (4/5) of cervical cancer tissues were Stage I, II and III of the disease, respectively in SC. Exceptionally one case of HPV16 was found at Stage II of AC (20%). However, samples found positive for HPV18 or dually positive for HPV16 and HPV18, were classified at advanced stages of SC (60%, 40% Stage III respectively) and AC (80%, 20% Stage II, respectively) (Figure 2).

Prevalence of oncogene-derived transcripts for HPV16 compared with HPV16 DNA L1 finding

All samples were positive for the RNA control, β-actin, used to avoid false-negative results that could be due to degradation of RNA. Using the RT-PCR method, the major E6 and E7 HPV16 transcripts were detected in 22 of the 44 (50%) cancer cases (Table 4). The transcriptional activity of these oncogenes shows different levels of expression. The discrepancy in finding HPV16L1 DNA and HPV16 RNA E6-E7 (PCR and RT-PCR data) was not statistically significant.

Discussion

Epidemiological and molecular studies have shown that cervical infection by certain types of human papillomavirus is the precursor event in the genesis of cervical

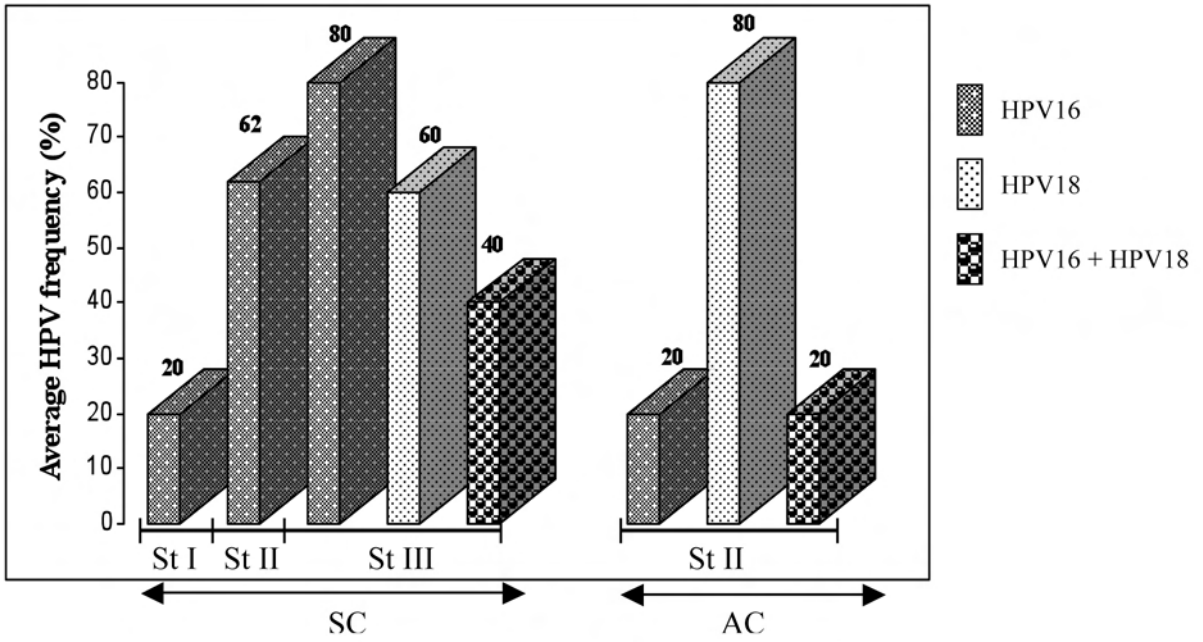

Figure 2. — Frequency distribution of HPV DNA types in different histological types and stages of cervical carcinoma. Abbreviations: St: Stage; SC: Squamous Carcinoma, AC: Adenocarcinoma.

neoplasia [17], and identification of HPV genotypes in clinical specimens is an important prognostic indicator for clinical screening and disease management [18]. Walboomers *et al*. [19] reported that the association between HPV and cervical cancer is high and up to 99.7%. Since papillomaviruses are difficult to culture and poorly detected by serological assays, molecular techniques remain the gold standard to detect the presence [12]. HPV PCR, a frequently used diagnostic tool for epidemiological investigations, involves amplification of HPV DNA by primers that bind to highly conserved regions within the L1 open reading frame of all genital HPV genotypes [20]. We wanted to rule out the possibility that the HPV late 1 region did not integrate into the cellular genomic DNA, and therefore could not be detected by PCR. Our findings demonstrated that HPV16 was the most prevalent type among HPV positive specimens (38/88), and HPV 18 (7/88) was the second. These results agree with the reported association of these genotypes with malignancy [21-24]. Moreover, our data showed that HPV16 was distributed differently in biopsies and smears with a rate of 54.5% versus 31.8%, respectively. False-negative rates for cervical premalignant lesions and cervical cancer in smears can be explained by the fact that the initial infection requires access of infectious particles to the cell in the basal layer [25] and therefore could not be detected. The prevalence of multiple HPV infections is a common phenomenon that can have clinical significance. In our study group 6.8% of cervical cancer specimens were found dually positive for HPV16 and HPV18. This rate is lower than what has been reported in recent studies showing rates between 9% and 32%, depending on ethnicity [26, 27]. Patients with multiple HPV types may have a higher risk of persistent infection compared to those with a single HPV type [28]. Persistent HPV infection, in turn, is necessary for the development of cervical cancer [29, 30]. In our study, we found that DNA yield recuperated varied widely. In fact, the quantity of DNA affects molecular detection; therefore, it represents a limitation for PCR assay. The higher positivity for HPV was found in biopsies having the highest amounts of DNA. This affectation could also be related to the viral type since this limiting factor was seen especially for HPV18 detected in biopsies with a high DNA amount, however with a lesser quantity of DNA extracted from smears HPV18 was lacking. This result is also consistent with Prétet *et al.'s* report. Their study confirmed that the presence of HPV is significantly associated with viral load [31]. In relation to HPV16 and 18 distributions according to the histological type we found statistically significant differences ($p = 0.005$). These findings are in accordance with several previous studies that have consistently found HPV 16 to be preferentially associated with SC rather than AC of the cervix [8]. HPV 18 has been associated with more aggressive forms of cervical intraepithelial neoplasia, invasive cervical cancer (adeno- and adenosquamous carcinomas) higher genome integration rate, and a greater likelihood of cancer recurrence and lymph node metastasis [32, 33]. We agree with other reports on concluding that HPV18 is strongly associated with AC of the cervix, which rapidly progresses through the preinvasive stages of cervical neoplasia [34, 35]. HPV18 plays a relatively minor role (15.9%) among HPV infection in tumor biopsies, whereas it was absent in

smears. Hence, cytological screening entails substantially lower protection against AC than SC of the cervix. A possible explanation could be that HPV18 infections preferentially increase in cervical AC and more often are localized in the endocervical canal [36, 37]. We agree with others who have concluded [38] that the use of smears might lower the sensitivity of HPV analyses and thus underestimate the true HPV prevalence in our cohort. The proportion of both HPV types clearly augments in accordance with the stage of cervical carcinoma. This is supported by previous data of Schelcht *et al*., where the presence of HR-HPV types was higher as the lesion evolved [39]. In the same way, such multiple HPV infections are frequently in advanced stages and have recently been detected in invasive cervical cancer [26, 27]. After screening cervical carcinoma specimens, the rate of HR-HPV DNA types detection was found to be 31.8% and 63.6%, respectively in smears and biopsies. On this basis, our results prompted us to focus on mRNA HPV16 E6/E7 expression in biopsies, of which expression is required for maintenance of malignancy. There are only minor differences between the mRNA E6-E7 and DNAL1 HPV16 detection rates. It has been shown that E7 promotes the formation of benign lesions whereas E6 works to complete the malignant transformation [40]. Cuschieri *et al*. recently reported that the detection of E6 or E7 transcripts in baseline samples helped predict those patients who were likely to carry a persistent infection [41]. Further elucidation of these findings utilizing quantity of mRNA could be more revealing. Therefore HPVL1 PCR is especially useful in screening, while the detection of oncogene transcripts could serve as a marker for risk of the development of cervical cancer.

Conclusions

In summary, this study provides further insights into Tunisian cervical cancer specimens with implications of HPV-based prevention strategies. Further investigations will be required to define more precisely the impact of practical conditions on the quality of viral genome, and on viral revelation. Nevertheless, type-specific HPV testing is valuable to address the burden of HPV infections epidemiologically and to gain more insights into the natural history and dynamics of HPV infections. To further establish the potential of HPV E6 and E7 mRNA other promising biomarker molecules that regulate these viral oncogene expressions will be predictive for prevention, early diagnosis, and treatment for cervical carcinoma.

References

[1] Parkin D.M., Bray F., Ferlay J., Pisani P.: "Global cancer statistics, 2002". *CA Cancer J. Clin*., 2005, *55*, 74.

[2] Maalej M., Mrad K., Kochbati L., Guigua A., Ben Abdallah M., Ben Ayed F., Ben Romdhane K.: "Cervical cancer in Tunisia: an epidemiological, clinical and pathological study". *Eur. J. Obstet. Gynecol. Reprod. Biol*., 2004, *113*, 226.

[3] Kim S.W., Yang J.S.: "Human Papillomavirus type 16 E5 protein as a therapeutic target". *Yonsei Med. J*., 2006, *47*, 1.

[4] Lagunas-Martinez A., Madrid-Marina V., Gariglio P.: "Modulation of apoptosis by early human papillomavirus proteins in cervical cancer". *Biochim. Biophys. Acta*, 2010, *1805*, 6.

[5] Sánchez-Anguiano L.F., Alvarado-Esquivel C., Reyes-Romero M.A., Carrera-Rodriguez M.: "Human papillomavirus infections in women seeking cervical Papanicolaou cytology of Durango, Mexico: prevalence and genotypes". *BMC Infect. Dis*., 2006, *6*, 27.

[6] McMurray H.R., Nguyen D., Westbrook T.F., McAnce D.J.: "Biology of human papillomaviruses". *Int. J. Exp. Pathol*., 2001, *82*, 15.

[7] Yoshida T., Sano T., Oyama T., Kanuma T., Fukuda T.: "Prevalence, viral load, and physical status of HPV 16 and 18 in cervical adenosquamous carcinoma". *Virchows Arch*., 2009, *455*, 253.

[8] Im S.S., Wilczynski S.P., Burger R.A., Monk B.J.: "Early stage cervical cancers containing human papillomavirus type 18 DNA have more nodal metastasis and deeper stromal invasion". *Clin. Cancer Res*., 2003, *9*, 4145.

[9] Abudukadeer A., Ding Y., Niyazi M., Ababaikeli A., Abudula A.: Distribution of HPV genotypes in uterine cervical lesions among the Uighur women in Xinjiang province of China. *Eur J. Gynaecol. Oncol.,* 2010, *31*, 315.

[10] Bory JP., Cucherousset J., Lorenzato M., Gabriel R., Quereux C., Birembaut P., Clavel C.: "Recurrent human papillomavirus infection detected with the hybrid capture II assay selects women with normal cervical smears at risk for developing high grade cervical lesions: a longitudinal study of 3,091women". *Int. J. Cancer*, 2002, *102*, 519.

[11] Lie A.K., Risberg B., Borge B., Sandstad B., Delabie J., Remala R. *et al*.: "DNA-versus RNA-based methods for human papillomavirus detection in cervical neoplasia". *Gynecol. Oncol*., 2005, *97*, 908.

[12] Albrecht V., Chevallier A., Magnone V., Barbry P., Vandenbos F., Bongain A. *et al*.: "Easy and fast detection and genotyping of high-risk human papillomavirus by dedicated DNA microarrays". *J. Virol. Methods*., 2006, *137,* 236.

[13] zur Hausen H.: "Papillomaviruses and cancer: from basic studies to clinical application". *Nat. Rev. Cancer,* 2002, *2*, 342.

[14] Ma B., Xu Y., Hung C.F., Wu T.C.: "HPV and therapeutic vaccines: where are we in 2010?". *Curr. Cancer Ther. Rev*., 2010, *6*, 81.

[15] Yang Y.Y., Koh L.W., Tsai J.H., Tsai C.H., Wong E.F., Lin S.J., Yang C.C.: "Correlation of viral factors with cervical cancer in Taiwan". *J. Microbiol. Immunol. Infect*., 2004, *37*, 282.

[16] Sima N., Wang S., Wang W., Kong D., Xu Q., Tian X. *et al*.: "Antisense targeting human papillomavirus type 16 E6 and E7 genes contributes to apoptosis and senescence in SiHa cervical carcinoma cells". *Gynecol. Oncol*., 2007, *106*, 299.

[17] Franco E.L.: "Cancer causes revisited: human papillomavirus and cervical neoplasia". *J. Natl. Cancer Inst*., 1995, *87,* 779.

[18] Muñoz N., Mendez F., Posso H., Molano M., van den Brule A.J., Ronderos M. *et al*., Instituto National de Cancerologia HPV Study Group: "Incidence, duration, and determinants of cervical human papillomavirus infection in a cohort of colombian women with normal cytological results". *J. Infect. Dis*., 2004, *190*, 2077.

[19] Walboomers J.M., Jacobs M.V., Manos M.M., Bosch F.X., Kummer J.A., Shah K.V. *et al*.: "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide". *J. Pathol*., 1999, *189*, 12.

[20] Castle P.E., Lorincz A.T., Mielzynska-Lohnas I., Scott D.R., Glass A.G., Sherman M.E. *et al*.: "Results of human papillomavirus DNA testing with the hybrid capture 2 assay are reproducible". *J. Clin. Microbiol*., 2002, *40*, 1088.

[21] Bosch F.X., Lorinecz A., Muñoz N., Meijer CJ., Shah KV.: "The causal relation between human papillomavirus and cervical cancer". *J. Clin. Pathol*., 2002, *55*, 244.

[22] Clifford G.M., Smith J.S., Plummer M., Munoz N., Franceschis S.: "Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis". *Br. J. Cancer*, 2003, *88*, 63.

[23] Thomas J.O., Herrero R., Omigbodun A.A., Ojemakinde K., Ajayi I.O., Fawole A. *et al*.: "Prevalence of papillomavirus in women in Ibadan, Nigeria: a population-based study". *Br. J. Cancer*, 2004, *90*, 638.

[24] Dictor M., Warenholt J.: "Single-tube multiplex PCR using type-specific E6/E7 primers and capillary electrophoresis genotypes 21 human papillomaviruses in neoplasia". *Infect. Agent. Cancer*, 2011, *6*, 1.
[25] Pittayakhajonwut D., Angeletti P.C.: "Viral trans-factor independent replication of human papillomavirus genomes". *Virol. J.*, 2010, *7*, 123.
[26] Herrero R., Hildesheim A., Bratti C., Sherman M.E., Hutchinson M., Morales J. *et al.*: "Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica". *J. Natl. Cancer Inst.*, 2000, *92*, 464.
[27] Huang L.W., Chao S.L., Chen P.H., Chou H.P.: "Multiple HPV genotypes in cervical carcinomas: improved DNA detection and typing in archival tissues". *J. Clin. Virol.*, 2004, *29*, 271.
[28] Chaturvedi A.K., Katki H.A., Hildesheim A., Rodríguez A.C., Quint W., Schiffman M. *et al.*: "Human Papillomavirus Infection with Multiple Types: Pattern of Coinfection and Risk of Cervical Disease". *J. Infect. Dis.*, 2011, *203*, 910.
[29] Nobbenhuis M.A.E., Walboomers J.M.M., Helmerhorst T.J.M., Rozendaal L., Remmink A.J., Risse E.K. *et al.*: "Relation of human papillomavirus status to cervical lesions and consequences for cervical-cancer screening: a prospective study". *Lancet*, 1999, *354*, 20.
[30] Twu N.F., Yen M.S., Lau H.Y., Chen Y.J., Yu B.K., Lin C.Y.: Type-specific human papillomavirus DNA testing with the genotyping array: a comparison of cervical and vaginal sampling. *Eur. J. Obstet. Gynecol. Reprod. Biol.,* 2011, *156*, 96.
[31] Prétet J.L., Dalstein V., Monnier-Benoit S., Delpeut S., Mougin C.: "High risk HPV load estimated by Hybrid capture II correlates with HPV16 load measured by real-time PCR in cervical smears of HPV16-infected women". *J. Clin. Virol.*, 2004, *31*, 140.
[32] Burk R.D., Terai M., Gravitt P.E., Brinton L.A., Kurman R.J., Barnes W.A. *et al.*: "Distribution of human papillomavirus type 16 and 18 variants in squamous cell carcinomas and adenocarcinomas of the cervix". *Cancer Res.*, 2003, *63*, 7215.
[33] Arias-Pulido H., Peyton C.L., Torrez-Martinez N., Anderson D.N., Wheeler C.M.: "Human papillomavirus type 18 variant lineages in United States populations characterized by sequence analysis of LCR-E6, E2, and L1 regions". *Virology*, 2005, *338*, 22.
[34] Bray F., Carstensen B., Moller H., Zappa M., Zakelj M.P., Lawrence G. *et al.*: "Incidence trends of adenocarcinoma of the cervix in 13 European countries". *Cancer Epidemiol. Biomarkers Prev.*, 2005, *14*, 2191.
[35] Murillo R., Molano M., Martínez G., Mejía J.C., Gamboa O.: "HPV prevalence in Colombian women with cervical cancer: implications for vaccination in a developing country". *Infect. Dis. Obstet. Gynecol.*, 2009, *2009*, 653598.
[36] Bulk S., Berkhof J., Bulkmans N.W., Zielinski G.D., Rozendaal L., van Kemenade F.J. *et al.:* "Preferential risk of HPV16 for squamous cell carcinoma and of HPV18 for adenocarcinoma of the cervix compared to women to normal cytology in the Netherlands". *Br. J. Cancer*, 2006, *94*, 171.
[37] Kudelova M., Krivos V., Raslova H., Valovicova M., Belvoncikova P., Matis J.: "Occurrence of Human papillomavirus 16 and 18 in smears from the two cervix regions of onco-gynecological patients in Slovakia". *Acta Virol.*, 2008, *52,* 17.
[38] Kuramoto H., Sugimoto N., Iida M.: "Screening for cancer of the cervix with simultaneous pap smear and colposcopy". The efficacy of pap smear and colposcopy. *Eur. J. Gynaecol. Oncol.,* 2011, *32*, 73.
[39] Schlecht N.F., Kulaga S., Robitaille J., Ferreira S., Santos M., Miyamura R.A. *et al.*: "Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia". *JAMA*, 2001, *286*, 3106.
[40] Song S., Liem A., Miller J.A., Lambert P.F.: "Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis". *Virology*, 2000, *267*, 141.
[41] Cuschieri K.S., Whitley M.J., Cubie H.A.: "Human papillomavirus type specific DNA and RNA persistence-implications for cervical disease progression and monitoring". *J. Med. Virol.*, 2004, *73*, 65.

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