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Physical State of Human Papillomavirus Type 16 in Cervical Cell DNA*

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Multiplex PCR with specific primers for E2/E6 genes was used to assess the viral integration status of HPV-16 in women with low and high grade squamous intraepithelial lesions (LSIL and HSIL, respectively) in comparison to cervical cancer patients. Women with confirmed HPV-16 infection were examined: 30 with LSIL, 12 with HSIL and 23 with cervical cancer. The PCR products were separated electrophoretically in agarose gels and densitometric analysis was performed using Bio-Rad Quantity One software. E2 and E6 sequences of HPV-16 were detected in 91% of the women. The free episomal viral genome was not detected in the cervical carcinoma group. Twenty six percent of the samples obtained from this group harboured the integrated form, whereas the remaining samples possessed a mixture, i.e. episomal and integrated forms of viral DNA. The free episomal form dominated in women with LSIL and HSIL. In 6 cases the episomal and integrated forms were detected simultaneously. HPV-16 integration occurred in a subset of LSILs and HSILs, not only in the cervical cancer patients and correlated with progression of cytological changes. The assessment of the status of HPV-16 may be the molecular factor preceding the morphological features leading to malignancy.

Key words: Human papillomavirus genes, HPV-16, malignancy.

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Cervical cancer is one of the most frequent female malignant tumours and more than 500 000 new cases are reported each year all over the world. The etiological association between human papillomaviruses (HPV) and cervical cancer is now well established. Among 100 types of HPV discovered to date, about 30 are involved in anogenital infections. Epidemiological and biochemical data support the division of HPVs into two groups: high-risk, such as HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, capable of causing a progression to cancer of the uterine cervix, and low-risk, such as HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81, rarely, if ever, leading to cancer (ZUR HAUSEN 2000; MUNOZ *et al*. 2003; BOSCH *et al*. 2002). HPV-16 is the most extensively studied HPV type and serves as an important model for studying viral carcinogenesis (ZUR HAUSEN 2002). The E6 and E7 genes of high-risk HPV are known to play an important role in cervical carcinogenesis because they are highly expressed in cervical carcinoma cells and they have the ability to transform and immortalize primary keratinocytes (WOODWORTH *et al*. 1989; MUNGER *et al*. 1989). In the process of inactivation of tumour-suppressor gene products, the E6 proteins lead to the degradation of the p53 protein, and the binding complex of E7 and pRB proteins disturb the normal cell cycle and maintain abnormal cellular proliferation (SCHEFFNER *et al*.1990; HECK *et al*. 1992).

During a common infection and in most premalignant lesions, HPV is in an episomal state. However, most cervical carcinomas and derived cell

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lines maintain the HPV genome in an integrated form or in both integrated and episomal forms. Thus, integration into the chromosomes of the host cell has been proposed as an activation mechanism for progression from preinvasive lesions to cervical cancer. The genome integration of HPV usually disrupts or deletes E2 gene open reading frames, or rarely E1 open reading frames, which results in the loss of expression of the corresponding gene products. Disruption of these genes also leads to overexpression of the E6 and E7 oncoproteins, since the E2 gene products can repress activities from the HPV promoters that direct the expression of the E6 and E7 genes (ZUR HAUSEN 2002). The disruption of the E2 gene will cause the absence of the E2 gene sequences in the PCR product following integration.

Our study was undertaken to determine the physical status of HPV-16 by analyzing the ratios of E2 and E6 genes by multiplex PCR and to assess the viral integration of HPV-16 in cervical cell DNA from women with different grades of squamous intraepithelial lesions in comparison to cervical cancer patients.

Material and Methods

Sixty five HPV-16 positive cervical smears were classified by cytological examinations as low grade squamous intraepithelial lesions – LSIL (n=30), high grade squamous intraepithelial lesions – HSIL (n=12) and cervical carcinoma (n=23). Material was sampled from 20-75 year old women (average age for LSIL woman 36 years, for HSIL – 43 years and for cervical carcinoma 48 years). Specimens were collected in Digene Specimen Transport Medium (Digene Diagnostic, USA) and stored frozen at –70°C until processed.

DNA isolation

DNA was isolated using the Genomic DNA Prep Plus kit (A&A Biotechnology, Poland). Cell samples were lyzed in buffer with proteinase K (20mg/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 10mM TRIS-HCl pH 8,5 (preheat to 75°C).

PCR

HPV-16 detection was performed using the SPF10 primers in order to amplify a 65 bp fragment from the L1 region of the HPV genome and was followed by reverse hybridization (INNO-LiPA, Innogenetics, Belgium).

Multiplex PCR

The E2 and E6 genes of HPV-16 were detected by multiplex PCR using 2 pairs of primers synthesized according toYOSHINOUCHI (YOSHINOUCHI *et al.* 1999).

The primers for each sequence were:

5'-CTTGGGCACCGAAGAAACAC-3',

5'-TTGGTCACGTTGCCATTCAC-3' for the E2 gene and

5'-AAGGGCGTAACCGAAATCGGT-3',

5'-CATATACCTCACGTCGCAG-3' for the E6 gene

The amplicon sizes were 352 bp and 208 bp for the E2 and E6 sequences, respectively. PCR was performed in a final reaction volume of 25 μl, containing PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM of each primers, 2.5 U Ampli Taq Gold DNA polymerase (Perkin Elmer, USA) and template DNA (1 μg). The amount of isolated DNA was estimated spectrophotometrically. DNA extracted from cervical carcinoma cell line CaSki was used as positive control. The PCR conditions were as follows: initial cycle – 95°C for 5 min, 52°C for 1 min, 72°C for 1 min; the following 30 cycles: 95°C for 1 min, 52°C for 1 min, 72°C for 1 min; the final cycle – 95°C for 1 min, 52°C for 1 min, 72°C for 8 min.

The PCR products were separated electrophoretically in 2% agarose gels. Transilluminated gel images were digitalized and analyzed by the Gel Doc 2000 documentation system (Bio-Rad, USA). Values corresponding to the intensity and density of the gel bands of different PCR products were obtained with the help of Quantity One 4.0 software (Bio-Rad, USA). The ratio of E2 to E6 copy numbers was calculated to determine the physical status of the HPV-16 viral gene. HPV-16 in pure

Table 1

Correlation between physical status of HPV-16 DNA and cytological diagnoses

Cytology	Physical status of HPV-16 DNA		
	Episomal E2/E6≥1	Episomal and integrated 1>E2/E6>0	Integrated E2/E6=0
LSIL n=30	28	2	0
HSIL n=12	8	4	0
Ca* n=23	0	17	6

Ca* – cervical carcinoma

episomal form was expected to have equivalent copy numbers of E2 and E6 genes (E2/E6 ratio> 1 or =1), whereas preferential disruption of E2 upon viral integration should result in less E2 gene copies than E6. This means that an E2/E6 ratio of less than 1 would indicate the presence of both integrated and episomal forms, while a ratio of 0 would indicate the presence of integrated form only.

Results and Discussion

A total of 65 samples were examined in which HPV-16 was previously confirmed by the SPF-10 primer set and reverse hybridization. Several techniques have been described for the elucidation of HPV-16 status in infected cells, such as Southern blot, real-time PCR and two dimensional gel electrophoresis (PARK *et al.* 1997; NAGAO *et al.* 2002; HUDELIST *et al.* 2004). In this study we used multiplex PCR with primers specific for E2 and E6 genes and analyzed the quantitative ratio of PCR products.

HPV-16 E6 was found in all specimens. The E2 sequence of HPV-16 was detected in 59 (91%) samples, suggesting that in the remaining negative samples the virus may have been in integrated form only. The physical status of HPV-16 in samples with different cytological results are shown in Table 1. In women with LSIL and HSIL, the episomal form dominated (93% and 67%, respectively, with E2/E6 ratio>1 or =1). In 6 cases the episomal and integrated forms of HPV-16 were detected simultaneously. In material obtained from patients with cervical cancer the E2 region was amplified in 17 (74%) of cases. In this group 26% of patients exclusively harboured the integrated form (E2/E6 ratio = 0). In women with cervical carcinoma no free episomal HPV-16 was found but only mixed (1>E2/E6 ratio>0) or integrated forms of the viral genome. The examinations showed a tendency of increasing frequency of HPV-16 integration with progression to malignancy. We confirmed also that HPV-16 integration occurred in a subset of LSILs and HSILs, not only in the cervical cancer specimens. This may suggest that viral integration may occur earlier than the onset of morphological changes, which could indicate a high grade lesion. The assessment of HPV-16 status may be a helpful complementary tool for cytological study in cervical screening to identify women at risk for developing high grade squamous intraepithelial lesions or carcinoma.

References

BOSCH F. X., LORINCZ A., MUNOZ N., MEIJER C. J., SHAH K. V. 2002. The causal relation between human papillomavirus and cervical cancer. J.Clin.Pathol. **55**: 244-265.

HECK D. V., YEE C. L., HOWLEY P. M., MUNGER K. 1992.Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomavirus. Proc. Natl. Acad. Sci. USA **89**: 4442-4446.

HUDELIST G., MANAVI M., PISCHINGER K., WATKINS-RIEDEL T., SINGER C. F., KUBISTA E., CZERWENKA K. F. 2004. Physical state and expression of HPV DNA in benign and dysplastic cervical tissue: different levels of viral integration are correlated with lesion grade. Gynecol. Oncol. **92**: 873-880.

MUNGER K., PHELPS W. C., BUBB V., HOWLEY P. M., SCHLEGEL R. 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation primary human keratinocytes. J. Virol. **63**: 4417-4421.

MUÑOZ N., BOSCH F. X, DE SANJOSE S., HERRERO R., CASTELLSAGUE X., SHAH K. V., SNIJDERS P. J. F., MEIJER C.J. L. M. 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N. Engl. J. Med. **34**8: 518-527.

NAGAO S., YOSHINOUCHI M., MIYAGI Y., HONGO A., KODAMA J., ITOH S., KUDO T. 2002. Rapid and sensitive detection of physical status of human papillomavirus type 16 DNA by quantitative real-time PCR. J. Clin. Microbiol. **40**: 863-867.

PARK J., HWANG E., PARK S., AHN H., UM S., KIM C., KIM S., NAMKOONG S. 1997. Physical status and expression of HPV genes in cervical cancers. Gynecol. Oncol. **65**: 121-129.

SCHEFFNER M., WERNESS B. A., HUIBREGTSE J. M., LEVINE A. J., HOWLEY P. M. 1990. The E6 oncoprotein encoded by human papillomavirus type 16 and 18 promotes the degradation of p53. Cell **63**: 1129-1136.

WOODWORTH C. D., DONIGER J., DIPAOLA J. A. 1989. Immortalization of human keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. J. Virol. **63**: 159-164.

YOSHINOUCHI M., HONGO A., NAKAMURA K., KODAMA J., ITOH S., SAKAI H., KUDO T. 1999. Analysis by multiplex PCR of the physical status of human papillomavirus type 16 DNA in cervical cancer. J. Clin. Microbiol. **37**: 3514-3517.

ZUR HAUSEN H. 2000. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J. Natl. Cancer Inst. **92**: 690-698.

ZUR HAUSEN H. 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat. Rev. Cancer **2**: 342-350.