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TOMASZ POCHYLSKI [1], ANNA KWAŚNIEWSKA [2]

[1] Medical Center Oslo,
Vetlamsdon 101, 1405 Oslo, Norway
[2] Department of Gynaeceology and Obstetrics, University School of Medicine,
ul. Staszica 16, 20-081 Lublin, Poland

Prevalence of human papillomavirus in high grade dysplasia and squamous cell cervical cancer in women in Norway

Częstość występowania wirusów brodawczaka ludzkiego w dysplazji dużego stopnia i raku płaskonabłonkowym szyjki macicy u kobiet w Norwegii

SUMMARY

The causal relationship between subtypes of human papillomavirus (HPV) and cervical cancer has been firmly established. The epidemiological and clinical data are compelling and consistent, suggested that human papillomaviruses, especially HPV 16 and 18, play major role in the etiology of cervical cancer. The aim of this study was evaluation of the frequency of occurrence of human papillomavirus DNA in epithelium of squamous cell cervical carcinomas and high grade dysplasia of the cervix (CIN III). The study group consisted of 35 postoperative tissues from patients diagnosed with high grade dysplasia, 29 postoperative tissues from patients diagnosed with squamous cell cervical carcinoma. The control group consisted of normal cervical tissue specimens obtained from 33 patients that underwent hysterectomy due to uterine leiomyomas. Identification of DNA HPV type 16 and 18 was performed by means of PCR technique. The frequency of occurrence of HPV infection was statistically significantly higher in the examined group than that in the control group. Statistically significant correlation ($p<0.001$) provided an evidence for a direct relationship between infection with oncogenic types of HPV and both high grade dysplasia and squamous cell cervical carcinoma.

STRESZCZENIE

Wirusem szczególnie związanym z karcinogenezą szyjki macicy jest wirus brodawczaka ludzkiego (human papillomavirus — HPV). Obecność sekwencji genomu wirusa w stanach przednowotworowych, rakach szyjki macicy jak również w histologicznie prawidłowej szyjce macicy wskazuje, że wirus ten jest jednym z głównych czynników biorących udział w procesie nowotworzenia szyjki macicy. Celem pracy była ocena częstości występowania wirusów brodawczaka

ludzkiego w wybranej populacji kobiet norweskich z dysplazją dużego stopnia i rakiem płaskonabłonkowym szyjki macicy. Grupę badaną stanowiło 35 tkanek pooperacyjnych z rozpoznaniem dysplazji dużego stopnia oraz 29 tkanek pooperacyjnych z rozpoznaniem raka płaskonabłonkowego szyjki macicy. Grupą kontrolną były tkanki prawidłowej szyjki macicy uzyskane od 33 pacjentek operowanych z powodu mięśniaków macicy. Identyfikację DNA onkogennych wirusów HPV 16 i 18 przeprowadzono stosując metodę PCR. Częstość występowania infekcji HPV była statystycznie istotnie wyższa w grupie pacjentek badanych w porównaniu do grupy kontrolnej. Większa częstość występowania onkogennych typów HPV w przypadkach dysplazji dużego stopnia oraz raka płaskonabłonkowego szyjki macicy była statystycznie istotna ($p<0,001$).

Key words: HPV 16/18, high grade dysplasia, squamous cell cervical cancer.

INTRODUCTION

Every year approximately 400,000 new cases of cervical carcinoma are recorded worldwide. This type of cancer ranks third in the frequency of occurrence, constituting 9.8% of all cases of new malignant neoplasms in women (15). Incidence of cervical carcinoma is unevenly distributed in different geographical regions. In developed countries relative frequency of occurrence of this type of cancer is 4.4%, while in developing countries it is 15% (15).

Norway is one of the countries with the lowest mortality rate in Europe and the world, with the rate of approximately 4%, nowadays (10). This success has been achieved in Norway and other Scandinavian countries thanks to the introduction of screening tests and of an active screening program for cervical carcinoma in the 1980 s. Central Register of Neoplastic Diseases and National Program for Prophylactic Examinations started to operate in Norway in 1991. The register data show that, e.g. in 1998, squamous cell cervical carcinoma was diagnosed in 265 women and there were 50 cases of cervical adenocarcinoma. They constituted 4% of all registered cases of neoplasms in women. At this time 134 women died due to cervical carcinoma. The neoplasm occurred most frequently in older women and only sporadically in women below 25 years of age (10).

Every year about 2,000 cases of cervical intraepithelial neoplasia are detected (3). Early detection of this type of cancer in its preinvasive stage, that is in the stage of intraepithelial changes (CIN — cervical intraepithelial neoplasia), combined with early implementation of sparing surgery techniques has led to decrease of the number of new cases of cervical carcinoma in Norway from 21 cases per 100,000 people in 1974 (4) to 12 cases per 100,000 people in 2000 (10).

The causal relationship between subtypes of human papillomavirus (HPV) and cervical cancer has been firmly established. The epidemiological and clinical data are compelling and consistent, suggesting that human papilloma viruses infection, especially with type HPV 16 and 18, play major role in the etiology of cervical cancer (16).

The aim of this study was evaluation of the frequency of occurrence of human papillomavirus DNA in epithelium of squamous cell cervical carcinomas and high grade dysplasia of the cervix (CIN III).

MATERIAL AND METHODS

Material

The examined material consisted of 97 preserved tissues collected in the years 1986–1988 in Aker Sykeus hospital in Oslo from patients that underwent surgeries due to: 1) squamous cell cervical carcinoma, 2) high grade dysplasia and 3) uterine myoma. This material was obtained from the Chair of Pathological Anatomy of Aker Sykehus University Hospital in Oslo.

The tested group consisted of 35 postoperative tissues from patients diagnosed with high grade dysplasia including 16 tissues from carcinoma *in situ* and 29 postoperative tissues from patients diagnosed with squamous cell cervical carcinoma, in accordance with Burhardt and Ostor (5) and Richart (12) classification.

The control group consisted of histologically normal cervical tissue specimens obtained from 33 patients that underwent hysterectomy due to uterine leiomyomas.

As far as the histopathological type indicated, in the group of patients with squamous epithelial carcinoma 18 cases were diagnosed as *macrocellularae keratotodes*, 8 cases as *macrocellularae akeratodes* and 4 cases as *microcellularae akeratodes*.

In respect to differentiation of the neoplastic cells, the following groups of patients were distinguished: (G1 n = 5, G2 n = 14, G3 n = 10), according to the WHO gradation (13).

According to the FIGO (Federation Internationale de Gynecologie et Obstetrique) clinical staging system (9) 16 patients were classified as the stage 0 (*carcinoma in situ)*, 22 women as the IA, 6 as the IB, and 1 as the IIA stage.

There were no significant differences in mean age of women subjected to surgery due to squamous cell cervical cancer if compared to the control patients (49.93 $\pm$13.49 vs 48,94 $\pm$6.12). However, (statistical) mean of age of women with CIN III was lower than that of the women with squamous cell cervical cancer and those from the control group ($p<0.05$) (Table 1).

Table 1. Demographic characteristics in the studied and the control groups

Group	N	Mean	Standard deviation	Probability
High grade dysplasia	35	35.51	9.02	$p<0.001^{*,**}$
Squamous cell cervical carcinoma	29	49.93	13.49	$p<0.001^{***,a}$ n.s.
Control	33	48.94	6.12	
Total	97	44.03	12.09	

N — number of patients examined.

* — in relation to the squamous cell cervical carcinoma examined: value of the test function F = 6.268, p = 0.015; t = –5.453, $p<0.001$.

** — in relation to control: value of the test function F = 5.125, p = 0.027; t = –7.669, $p<0.001$.

*** — in relation to the high grade dysplasia group studied: value of the test function.

F = 19.746, $p<0.001$; t = 0.380, $p<0.001$.

^{a}n.s. no statistical significance as compared to the control group: value of the test function.

F = 19.346, p = 0.718, t = 364, p = 0.705.

Methods

Paraffin blocks with tissue fixed in 10% buffered formalin were cut into 4 μm thick pieces. The microtome was rinsed with alcohol before cutting each block. A new cutting blade was used for the cutting of each specimen. The pieces obtained in this manner were placed in a 1.5 ml polypropylene tube and paraffin was removed in xylene at 37°C for 30 min. Afterwards, they were centrifuged twice at 6000 rpm for 3 min, rinsed twice in 1 ml of absolute alcohol for 30 min and air dried.

After removal of the paraffin, the pieces were homogenised in l ml of Hirt buffer of the following composition: 0,01 M Tris-HCI pH 7.5; 0,01 M EDTA; 0.6% SDS.

The homogenate was incubated for 30 min at room temperature and digested with K proteinase (50 μg/ml for 24 hours at 37°C). Finally, 0.5 volume of mixture; phenol: chloroform: isoamyl

alcohol (25 : 24 : 1, v/v/v) was added. The mixture was shaken for 15 min at room temperature and centrifuged for 15 min at 3000 rpm. Subsequently, the equal volume of phenol, chloroform, isoamyl alcohol mixture was added to the water phase and after vigorous shaking subjected to centrifugation. The above treatment was repeated till complete DNA purification was achieved (manifested as lack of an interphase). Then, 0.5 volume of isopropylene alcohol and 0.1 volume of 3M potassium acetate (pH 7.0) was added to the water phase.

The air dried DNA samples obtained in the above manner were then rinsed in 80% ethanol and dissolved in distilled water. The samples were stored at –20°C.

Quantitative determination of DNA was carried out by the spectrophotometric method using an automatic spectrophotometer [Pharmacia Co]. In order to determine the amount of DNA an aliquot (1 μl) of the sample was dissolved in 69 μl of re-distilled water and measured after calibration of the spectrophotometer. After automatic processing of the data, the result was expressed in μg/ml.

HPV-PCR identification

In order to identify viral deoxyribonucleic acid in the DNA extracted from the post-operational materials isolated from the groups of patients examined (high grade dysplasia and squamous cell cervical cancer) and the control group, PCR was carried out using primers with sequences complementary to various types of the HPV virus (Table 2) (16). The degree of matrix purity of the complete human DNA isolated from the material studied was evaluated by means of primers for β-globins PC03 and PC04 (Table 2) (16).

Table 2. Primers for PCR study

Primers	Region of amplification	Sequence 5'–3'	Product size
MY09	L1	CGTCCMARRGGAWACTGATC	450 bp
MY11		GCMCAAGGWCATAAYAATGG	
		M = A+C, R = A+G, W = A+T, Y = C+T	
HPV16/L1 A	L1	GCCTGTGTAGGTGTTGAGGT	264 bp
HPV16/L1B		TGGATTTACTCCAACATTGG	
HPV18/L1 A	L1	GTGGACCAGCAAATACAGGA	162 bp
HPV18/L1B		TGCAACGACCACGTGTTGGA	
HPV18ME12	E6	CACGGCGACCCTACAAGCTACCTG	404 bp
HPV18ME50		TGCAGCACGAATTGGCACTGGCCTC	

In order to carry out chain polymerisation reaction, the following components were used: 1) deoxynucleotide-5'-triphosphate (dNTP) with a concentration of 200 μmol/dm^3 in the reaction mixture; 2) primers with concentration of 1 μmol; 3) 1 unit per 25 μl of Taq DNA polymerase [Promega]; 4) DNA template with concentration of about 10 ng/μl; 5) 100 mmol buffer Tris HC1 (pH = 8.8) [Boehringer Mannheim], KCl with a concentration of 500 mmol, 1% Triton X-100, and 15 mmol of $MgCl_2$.

The total volume of 10 μl of each PCR reaction was covered with 15 μl of mineral oil. The PCR reaction mixtures were preliminary denatured for 15 min at 94°C. Then the total of 31 cycles were run as follows 1) denaturation for 30 sec at 94°C, 2) annealing of the primers for 30 sec at 59°C, 3) extension for 60 sec at 72°C with a final extension step at 72°C for 420 sec.

The PCR products and HindI digested pBluscript as DNA size marker [Promega] were separated on 2% agarose gel and stained with ethidium bromide.

The negative controls consisted of PCR reactions containing all reagents without DNA template.

Statistical analysis

From the statistical analysis of the results obtained from HPV(+) and HPV(–) patients with squamous cell cervial cancer and high grade dysplasia of the uterine cervix, correlation tables containing structure indices were complied. A method of statistical interference, the verification of hypotheses based on homogeneity and independence test χ^2 was used. Statistical significance was found at $p<0.05$. The statistical analyses were made on IBM PC, using SPSS 8.0 PL for Windows 95, Statistica 5.0.

RESULTS

Utilization of a universal primer that allows for identification of viral DNA sequences from 33 HPV types showed that the frequency of latent infection in the control group was 24.2% (8/33). HPV 16 infection was found in the control group in 6.06% of patients (2/33) (Fig. 1), while HPV 18 only in one case, that constitutes 3.03% (Table 3).

Table 3. Frequency of HPV DNA occurrence in the studied and the control group of women

Group	HPV DNA type analysis						
	HPV DNA 33 types (universal primer)		HPV DNA type 16		HPV DNA type 18		probability
	N	%	N	%	N	%	
High grade dysplasia n = 35	28	80	14	40	4	11.43	$p<0.001^*$
Squamous cell cervical cancer n = 29	27	90	14	48.27	10	34.48	$p<0.001^{**}$
Control group n = 33	8	24.2	2	6.06	1	3.03	

N — number of patients.
* — in relation to control: ($\chi^2 = 21.196$, $p<0.001$, the Fisher test — $p<0.001$.
** — in relation to control: ($\chi^2 = 27.520$, $p<0.001$, the Fisher test — $p<0.001$.

In the group of 35 patients with histopathological high grade cervical dysplasia, 33 types of HPV were found in 80% of patients (28/35). Infection with HPV 16 constituted 40% (14/35) (Fig. 2) whereas HPV 18 was found in 11.43% of patients (4/35). In the group of patients with high grade dysplasia HPV 16 infections were found 3.5 times more frequently than HPV 18 infections (Table 3).

Among 29 specimens of tissue from women with squamous cell cervical cancer diagnosis, papillomavirus infections were found in 90% of patients (27/29). Type 16 infection was identified in 48.27% of patients (14/27) (Fig. 3), while type 18 in 34.48%. HPV 16 of HPV occurred 1.4 times more frequently than type 18 in the group of patients with squamous cell cervical carcinoma. Oncogenic type 18 of HPV was identified 2.5 times more often in tissue fragment from patients operated due to squamous cell cervical carcinoma compared to patients with high grade dysplasia.

The frequency of occurrence of HPV infection was statistically significantly higher in the examined group than that in the control group. Statistically significant correlation ($p<0.001$) is a proof for direct a strict connection between infection with oncogenic HPV types and high grade dysplasia and cervical carcinoma (Table 3).

DISCUSSION

The obtained results concerning the prevalence of HPV infections are consistent with data of other authors.

Olsen et al. (9) showed that in Norway HPV infections in a group of patients with CIN were similar to those described in this work and constituted 90.8%. However, in a control group the ratio of infections was much lower (15.4%) (OR: 72.8: 95% CI: 27.6–191.9; for HPV 16 OR: 182.4 95% CI: 54.0–616.1).

Long lasting epidemiological studies carried out in 22 countries (as a part of IBSCC program — International Biological Study on Cervical Cancer), using various methods, showed the presence of HPV DNA in 92.7% of invasive cervical lesions (11).

A study carried out by Bosch et al. (4) in Spain, on a group of 157 patients with high grade dysplasia and 193 control patients revealed that HPV infections in the examined group constituted 72.4% whereas in the control groups the latent infections with HPV types of high oncogenic risk occurred at a frequency of 4.7%. OR value for all types of HPV-DNA in the tested group was 56.9 (95% CI: 24.8–130.6), OR for HPV 16 was 295.5 (95% CI: 44.8–1946.4). Similar studies carried out by this author in Columbia showed that prevalence of HPV--DNA infections in a group of 125 patients was 63.2% in comparison to 10.5% in the controls (OR: 15.5%, 95% CI: 29.4–82, for HPV 16 OR: 27.1 95% CI: 10.6–69.5).

Becker (2) demonstrated that in a group of 176 American women with CIN II/III the prevalence of HPV infection was 93.8%, compared to 42.1% (OR: 20.8, 95% CI: 10.8–40.2) in 311 control patients. Schifman's studies (14), carried out

in the USA, exhibited 90% (OR: 42, 95% CI: 15.3–124.3; for HPV 16 OR: 180, 95% CI: 49–630) HPV infection of high oncogenic risk in a group of 50 patients with medium and high grade dysplasia.

Studies in Poland, concerning the prevalence of HPV infections in women with cervical dysplastic lesions have been carried out by Kwaśniewska (8) and Goździcka-Józefiak (7) in which the virological diagnostics was based on the method of Digene Hybrid Capture System I and the chain reaction of polymerase.

A comparative analysis of the current results with the previous data showed the high convergence of data in both the group examined and the control groups.

Application of polymerase chain reaction in this work for identification of DNA from human papilloma virus in the clinical material allowed to detect the infection with the latent form of virus. According to other authors, remission of latent forms of HPV infection, *e.i.* virus does not replicate can last form several to up to twenty years (16). Analysis of replication cycles of the HPV virus suggests that the state of latency is caused by a lack of factors enabling transcription and replication of the viral genome in host cell. This state can be also the result of the presence in the cell of specific repressors of the expression of genetic information (16).

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