Chlamydia trachomatis and *herpes simplex virus 2* infection in vulvar intraepithelial neoplasia associated with human papillomavirus

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Summary

Background: The role of viral and bacterial co-infection is stressed in VIN. A view that VIN is a sexually transmitted disease made the area of research larger and stimulated scientists to seek other sexually transmitted factors, among which *Chlamydia trachomatis* and *Herpes simplex* are frequently examined.

Purpose: The aim of the study was to evaluate the frequency of occurrence of HPV DNA and the frequency of co-infection with Herpes virus type 2 and *Chlamydia trachomatis* in VIN.

Material and Methods: We identified archival diagnostic phase tissue specimens from 41 cases of vulvar intraepithelial neoplasia III. From the same paraffin blocks containing material from the margins of surgical sections during vulvectomy, normal epithelial tissue fragments were collected. They constituted the control group. Lesion characteristics were examined in comparison with the presence of HPV DNA, HSV-2 and *Chlamydia trachomatsis.* Identification was performed using PCR.

Results: In the study group HPV infection was found in 75.6% of cases. In 73% of cases it was HPV 16. In the control group we found HPV 16 DNA in only one case (2.43%). In the HPV positive study group HPV 16 was found in 30 (30/31) cases. In only one case (1/31) it was HPV 18 type. In the study group of 41 cases with VIN, HSV-2 infection was found in six cases (14.63%). In comparison with the control group (9.75%) the difference was not statistically significant. The frequency of occurrence of *Chlamydia trachomatis* in the analyzed study material was 14.63% (6/41) and in the control group it was 9.75% (4/41). The difference was not statistically significant. Statistical analyses of correlations between the occurrence of DNA HPV and HSV-2 as well as of HPV and *Chlamydia trachomatis* showed no correlation in either case.

Conclusion: No correlation was found between the frequency of occurrence of HPV and HSV-2 and HPV and *Chlamydia trachomatis* in either group.

Key words: VIN; HPV; HSV-2; *Chlamydia trachomatis;* Vulvar intraepithelial neoplasia.

Introduction

While we know quite a lot about intraepithelial neoplasias of the cervix, the clinical course and molecular mechanisms of vulvar neoplasias are still relatively unknown [1]. Epidemiological data show that the risk factors for vulvar cancer are similar to the ones for cervical cancer [2]. Because the occurrence of intraepithelial cervical neoplasia and cervical cancer is etiopathologically connected with the presence of oncogenic types of HPV, it has also been suggested that there is a relation between this virus and the development of intraepithelial lesions within the vulva [2-4]. There are two types of VIN lesions: 1) classic (bowenoidal) VIN with positive HPV examination results and 2) simple vulvar intraepithelial neoplasia, occurring mainly in postmenopausal HPV-negative women [5-7].

Appreciating the influence of infection with oncogenic types of HPV on the development of VIN and vulvar carcinoma, early detection and treatment of coexisting infections is crucial [6, 7]. It is believed, that infections with herpes family viruses, especially HSV-2, and bacterial infections constitute factors promoting expression of oncogenic HPV proteins [8].

Thus the aim of the study was to evaluate the frequency of occurrence of HPV DNA and the frequency of co-infection with herpes virus type 2 and *Chlamydia trachomatis* in vulvar intraepithelial neoplasias.

Materials and Methods

We identified archival diagnostic phase tissue specimens from 41 cases of vulvar intraepithelial neoplasia III. Study material was obtained from patients who underwent surgery in the years 1992-2002 in hospitals in the Lublin region. Histopathological analysis of the material was performed in the Department of Pathomorphology of Skubiszewski University School of Medicine in Lublin. All vulvar specimens were reviewed by two pathologists (E.K. and M.Z.). The specimens were routinely fixed in 10% neutral-buffered formalin, embedded in paraffin and stained with hematoxylin-eosin for histological evaluation. The specimens chosen for evaluation were the ones which contained the largest amount of tissue with VIN III and the lowest possible amount of necrotic lesions. For this study, the tumors were reclassified in accordance with the criteria of the American Society of Clinical Pathologists [5-7].

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Sections for PCR were collected from each fragment. Eventually tissues from 41 cases of VIN III were qualified for the study group. From the same paraffin blocks containing material from the margins of surgical sections during vulvectomy, normal epithelial tissue fragments were collected. They constituted the control group. Lesion characteristics were examined in comparison with the presence of HPV DNA., HSV-2 and Chlamydia trachomatis.

Taking the presence of HPV DNA and histopathological examination results into account, VIN III cases were divided into two groups: 1) classic VIN, bowenoidal (average age 43.4) and 2) simple VIN (average age 67.1). The mean age of all patients with VIN was 49.5 years .

DNA extraction

Briefly, 5-8 μm paraffin-sectioned samples were placed in a microcentrifuge tube and 400 μl xylene was added and rotated vigorously for 2 min to deparaffinize tissue samples. After removal of the paraffin, the pieces were homogenized with the addition of 1 ml Hirt buffer with 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. Then, K proteinase was added reaching its final concentration of 50 μg/ml and incubated for 24 hours at a temperature of 37°C. After the incubation was finished, 0.5 volume of phenol/chloroform/isoamyl alcohol (in a ratio of 25:24:1) mixture was added to the solution; it was shaken for 15 min at room temperature and centrifuged for 15 min at 3000 rpm. A 0.5 volume of phenol, chloroform, isoamyl alcohol mixture was again added to the water phase obtained; it was shaken energetically and afterwards centrifuged. The above activities were repeated till complete purification of DNA, manifested as lack of an interphase, was achieved. Then, 0.5 volume of isopropylene alcohol and 0.1 volume of 3M acetate with pH 7.0 were added to the water phase obtained.

The DNA samples obtained in this manner were then rinsed in 80% ethanol and dissolved in distilled water after drying. The samples with dissolved DNA were stored at a temperature of -20°C.

Quantitative determination of the DNA obtained was carried out by the spectrophotometric method using an automatic spectrophotometer manufactured by Pharmacia Co. In order to determine the amount of DNA in a given sample, 1 μl of the sample was dissolved in 69 μ1 of redistilled water and after calibration of the spectrophotometer was placed in its measuring chamber. After automatic processing of the data measured, the result was read in μg/ml.

HPV- PCR identification

In order to identify viral genes in the DNA isolated from the study and control material, PCR analysis was performed using MY09/MY11 starters with sequences complementary to 33 HPV types, HPV16 L1/L2 starters complementary to HPV 16 as well as HPV18L1/L2 and HPV 18ME12/50 starters complementary to HPV 18. The degree of purity of the complete human DNA matrix isolated from the study material was evaluated using PC03 and PC04 starters for β-globin as a reference gene. The reaction was performed in the way described by Tuker *et al.* [9]. PCR was repeated in various variants using spermidine and increasing amounts of DNA purified in Qiagene columns. In statistical analysis, percentage of occurrence of the studied feature was given.

In order to carry out chain polymerization reaction, the following components were used: 1) deoxinucleotide-5'-triphosphate (dNTP) with a concentration of 200 μmol/dm^3 in the reaction mixture; 2) starter sections (primers) with a sequence characteristic of viruses from the HPV group with a concentration of 1 μmol/dm^3; 3) Taq polymerase of DNA with concentration of one unit per 25 μl; 4) DNA matrix with concentration of about 10 ng/μl; 5/buffer: Tris HC1 with pH = 8.8 and concentration of 100 mmol/dm^3, KC1 with a concentration of 500 mmol/dm^3, Triton X-100 1%, MgCL 15 mmol/dm^3.

The volume of the reaction mixture was 10 μl, and 15 μl of mineral oil was added to each sample. The samples were subjected to preliminary denaturation for 15 min at a temperature of 94°C, and then 31 amplification cycles were performed. Each of the cycles consisted of the following stages: 1) denaturation for 30 sec at 94°C, 2) starter connection for 30 sec at 59°C, 3) synthesis of complementary DNA for 60 sec at 72°C.

In the last cycle, the stage of complementary DNA synthesis (72°C) was extended to 420 seconds. When amplification was completed, PCR products were analyzed by electrophoretic separation in 2% agarose gel in the presence of a DNA size marker which was DNA pBluscript digested with a restrictive HindI enzyme.

Chlamydia trachomatis - PCR identification

PCR II was performed in order to identify bacterial deoxyribonucleic acid in the DNA isolated from postoperative material from the study and control groups. The product obtained in the first stage of PCR reaction was used as a matrix for PCR II (nested PCR). The size of the first-stage product was 116 bp, and of the second-stage product – 87 bp.

The temperature for seven individual stages of the second cycle, nested PCR, was: 95°, 54°, 72°, 95°, 54°, 72°, 4°, respectively. The time of duration of the individual stages: 4', 2', 2', 1', 2', 7', 2'; 35 cycles were performed. The time of duration of the whole program was 3 hr and 55 min.

Herpes virus type II PCR-identification

In order to identify viral DNA in the DNA isolated from postsurgical material from both the study and control groups, PCR was performed using HSV1 (5'-CATCACCGACCCGGAGAGGGAC -3') and HSV 2 (5'-GGGCCAGGCGCTTGTTGGTGA3-') (product size – 92bp) starters with sequences complementary to HSV (Herpes genitalis).

Statistical analysis

From the statistical analysis of the results obtained from HPV(+) and HPV(-) patients with VIN, correlation tables containing structured indices were compiled. A method of statistical interference, 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 an IBM PC, using SPSS 8.0 PL for Windows 95 and Statistica 5.0.

Results

Using Hart's classification [5, 6, 14] on our own studies performed to identify oncogenic HPV types we found the classic, bowenoidal type of VIN in 75.6% of analyzed cases. The simple VIN form occurred in 24.4% of analyzed cases (Table 1). In the study group HPV infection was found in 75.6% of cases. In 73% of cases it was HPV 16. In the control group we found HPV 16 DNA in only one case (2.43%) (Table 1).

In the HPV positive study group, 30 (30/31) cases were found to have HPV 16. In only one case (1/31) it was HPV 18 type. The difference in the frequency of occurrence of high-risk HPV DNA in the study group compared to the control group was statistically significant (Table 1).

In the study group of 41 cases with VIN, HSV-2 infection was found in six cases (14.63%). In comparison with the control group (4/41, 9.75%), the difference was not statistically significant. The frequency of occurrence of *Chlamydia trachomatis* in the analyzed study material was 14.63% (6/41) while it was 9.75% (4/41) in the control group. The difference was not statistically significant. Statistical analyses of correlations between the occurrence of HPV DNA and HSV-2 as well as HPV and *Chlamydia trachomatis* revealed no correlation in either case (Table 2).

Table 1. — *Frequency of DNA HPV occurrence in the studied and control groups of women.*

Group	DNA HPV universal		DNA HPV type 16		DNA HPV type 18		HSV-2		Chlamydia trachomatis	
	N	%	N	%	N	%	N	%	N	%
control group N = 41	1	2.43	1	2.43	0	0	4	9.75	4	9.75
case group N = 41	31	75.6*	30	73.10	1	2.43	6	14.63[a]	3	7.31[b]
VIN classic (bowenoidal) N = 31 (75.6%)	31	100.0**	30	96.77	1	4.16	5	16.12	3	9.67
VIN simple N = 10 (24.4%)	0	0	0	0	0	0	2	10.0	0	0.0

N: number of patients; *: in relation to the control: $\chi^2 = 24.596$, $p < 0.001$, the Fisher test -$p < 0.001$; **: in relation to the control group $\chi^2 = 28.420$, $p < 0.001$, the Fisher test -$p < 0.001$.
[a]: not statistically significant; [b]: not statistically significant.

Table 2. — *Correlation between the occurrence of HPV DNA and the frequency of occurrence of* Chlamydia trachomatis *and HPV as well as HSV-2 in VIN.*

Group	Relation	Statistical analysis
VIN N = 41	HPV and *Chlamydia trachomatis*	$\chi^2 = 0.343$, $p = 0.451$, n.s.
Control group N = 41	HPV and *Chlamydia trachomatis*	$\chi^2 = 3.234$, $p = 0.072$, n.s.
VIN N = 41	HPV and HSV-2	$\chi^2 = 0.243$, $p = 0.321$, n.s.
Control group N = 41	HPV and HSV-2	$\chi^2 = 3.234$, $p = 0.072$, n.s.

N: number of patients; n.s.: no statistical significance.

Discussion

Until recently there have been hardly any direct data confirming the fact that carcinogenesis related to HPV is the main factor in development of vulvar carcinoma. Indirect evidence for the existence of a cause-effect relationship is derived from the fact that HPV DNA and invasive vulvar carcinoma or VIN coexist in about 50-90% of cases [10]. The most frequent type of HPV in VIN is HPV 16. Types 18, 31, 33, 35 and 51 are much less frequent.

So far only one prospective study of young women has been performed [11]. Bjorge *et al.* [11] used serologic studies to show that the presence of HPV 16 at an early age is related to significantly higher risk of occurrence of vulvar carcinoma (RR = 5.0; CI: 1.1-22.0). It was also the first study confirming the possibility of a causative role of HPV in the development of VIN and vulvar carcinoma. Another study demonstrated a 5.3 times higher risk of occurrence of VIN in women, in whom there were antibodies against any virus-like particles of HPV 16 [12]. In the same study a stronger relationship was found between the presence of antibodies against HPV 16 and VIN III than the invasive vulvar carcinoma.

Not many prospective studies evaluating the influence of HPV on vulvar tissues have been performed [13, 14]. In one study 103 women with confirmed HPV infection in the region of the vulva were observed [14]. In one case, VIN III developed after two years.

In our study material, the frequency of occurrence of DNA of high oncogenic potential HPV types was 75.6% (31/41) in the study group and 2.43% (1/41) in the control group. Generally these values do not differ from frequencies observed by other researchers both in study and control groups, however, our results are a little lower than e.g., Hording *et al.'s* [14], who used the PCR technique to detect HPV 16 in 90% of VIN III cases. Our studies are also lower compared to results from a different region of Poland, where infections with oncogenic HPV types were found in 87% of patients with VIN. In these studies the histologically dominant type of VIN was bowenoidal [15]. However, our results are almost entirely consistent with the data given by Rodolakis *et al.* [16], who estimated the rate of HPV DNA occurrence at 75.2%. In this paper the mean age of the HPV-associated VIN patients was 46.3 (our study 43.4) compared to 52.8 years for those without (our study 67.1). Our results are comparable with van der Avoort *et al.* [17], who described HPV infection in VIN III in 71% and Hillemanns and Wang [18], who showed that 24 of 30 vulvar intraepthelial neoplasias were HPV positive (80%). They all used the PCR technique.

Repeated histopathological evaluation of the analyzed sections of tissues from patients who underwent surgery due to VIN III accompanied by PCR identification of HPV DNA allowed for making a distinction between classic and simple VIN III types. The classic type was diagnosed in 76% and the simple type in 24% of patients. This data is compatible with Hart's data [5, 6], which demonstrate more frequent occurrence of the classic VIN type. According to Hart [5] simple VIN is diagnosed in about 2-10% of cases of biopsy of VIN type lesions. The higher rate of diagnosed simple vulvar intraepithelial neoplasia in our study is the result of random selection of archival material.

Since HPV, HSV-2 and *Chlamydia trachomatis* are transmitted sexually and infect the same cell type, it has been postulated that viruses have the potential to interact with each other and play a role in neoplastic progression. Almost all the studies evaluating the role of HPV co-infection consider neoplastic lesions of the cervix. Epidemiological studies of the association of anogenital cancers with HPV and HSV-2 provide contradicting data. The Nordic cohort study of 550,000 women, in which the

authors re-evaluated the role of HSV-2 in cervical carcinogenesis by conducting a longitudinal, nested case-control study using 1974-1993 data and comparing the results with those from meta-analyses of studies, showed that HSV-2 did not play a role in cervical carcinogenesis [19]. Also in a study performed by Than *et al.* [20], although herpes simplex virus type 2 Bg/IIN transforms epithelial cells in vitro, it was not detected in cervical cancer specimens. However, one study by Smith *et al.* [21] remains in contradiction to the above cited studies. They demonstrated that HSV-2 infection may act in conjunction with HPV infection to increase the risk of invasive cervical carcinoma [21]. There are few studies analyzing co-infections in VIN. The ones available in the data bases (Medline, Pub Med) indicate a higher rate of HSV-2 and *Ch. trachomatis* in the studied cases.

Specimens from vulvar intraepthelial neoplasia were analyzed for the presence of herpes simplex virus-2 and HPV by Costa *et al.* [22]. A search for the HPV 16 E6 protein as well as the HSV-2 antigenic determinant LA1 and ICSP 11/12 protein was carried out with immunoperoxidase assay. Positivity to HSV-2 was observed in 33.3% of VIN cases. The results obtained by Costa *et al.* [22] are higher than the ones we acquired. It is most probably a result of the choice of study material and study method. In our previous studies evaluating the frequency of the occurrence of *Ch. trachomatis* and HSV-2 in vulvar smears using PCR we estimated the frequency of occurrence of HSV-2 as 3-4% [23].

When analyzing blood serum using serologic methods, most investigators indicate the presence of a relationship between HPV, HSV-2 and *Ch. trachomatis*. Studies of parrafin-embedded tissue or biopsies or cervical scrapings exclude such relationship. In 2004, Yang *et al.* [24] published a paper evaluating the correlation of viral factors with cervical cancer in paraffin-embedded tissue and frozen tissue. The correlation of HPV with cervical cancer was significantly different between frozen tissue and paraffin-embedded tissue. In the quoted paper, other viruses such as HSV-2 and CMV were not predictive of cervical cancer. They may not be in the oncogenic processes directly but might enhance the possibility of oncogenesis or infect cancer tissues opportunistically.

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References

[1] Flowers L.C., Wistuba E.A., Scurry J., Muller C.Y., Ashfaq R., Miller D.S. *et al.*: "Genetic changes during the multistage pathogenesis of human papillomavirus positive and negative vulvar carcinomas". *J. Soc. Gynecol. Invest.*, 1999, *6,* 213.

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

[3] Scheffner M., Werness B.A., Huibregtse J.M., Levine A.J., Howley P.M.: "The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53". *Cell.*, 1990, *63,* 1129.

[4] Munger K., Basile J.R., Duensing S., Eichten A., Gonzales S.L., Grace M., Zacny V.L.: "Biological activities and molecular targets of the human papillomavirus E7 oncoprotein". *Oncogene,* 2001, *20,* 7888.

[5] Hart W.R.: "Simplex (differentiated) VIN: an underappreciated threat". *Contemp. Obstet. Gynaecol.*, 2003, *48,* 59.

[6] Hart W.R.: "Vulvar intraepithelial neoplasia; historical aspects and current status". *Int. J. Pathol.,* 2001, *20,* 16.

[7] Hart W.R., Young R.H., Dail D.H.: "Tumors and related lesions of the female genital tract; based on the proceedings of the 56th Annual Anatomic Pathology Slide Seminar of the American Society of Clinical Pathologists". Dallas, ASCP Press, 1991.

[8] Meyers C., Andreansky S.S., Courtney R.J.: "Replication and interaction of herpes simplex virus and human papillomavirus in differencing host epithelial tissue". *Virology*, 2003, *315,* 43.

[9] Tucker R.A., Johnson P.R., Reeves W.: "Using the polymerase chain reaction to genotype human papillomavirus DNAs in samples containing multiple HPVs may produce inaccurate results". *J. Virol. Methods,* 1993, *43,* 321.

[10] IARC. Human Papillomaviruses. IRAC monographs on the evaluation of carcinogenic risks to humans. Vol. 64, Lyon, IARC, 1995.

[11] Bjorge T.B., Dillner J., Anttila T.: "Prospective sero-epidemiological study of role of human papillomavirus in non-cervical anogenital cancer". *Br. Med. J.,* 1997, *315,* 646.

[12] Hildesheim A., Han C.-L., Brinton L.: "Human papillomavirus type 16 and risk of pre-invasive and invasive vulvar cancer: results from a seroepidemiological case-control study". *Obstet. Gynecol.*, 1997, *90,* 748.

[13] Planner R.S., Hobbs H.B.: "Intraepithelial and invasive neoplasia of the vulva in association with human papillomavirus infection". *J. Reprod. Med.*, 1988, *35,* 503.

[14] Hording U., Junge J., Poulsen H., Lundvall F.: "Vulvar Intraepithelial Neoplasia II: a viral disease of undetermined progressive potential". *Gynecol. Oncol.,* 1995, *56,* 276.

[15] Adamek K., Szczudrawa A., Basta A., Wspólistnienie: "VIN i raka inwazyjnego sromu ze śródnablokową neoplazja i rakiem inwazyjnym szyjki macicy i lub pochwy, a infekcja HPV w obrębie dolnego odcinka narządu rodnego". *Gin. Pol.,* 2003, *9,* 657.

[16] Radolakis A., Diakomanolis E., Vlachos G., Iconomou Th., Protopappas A., Stefanidis C. *et al.*: "Vulvar intraepithelial neoplasia (VIN) - diagnostic and therapetic challenges". *Eur. J. Gynecol. Oncol.*, 2003, *24,* 317.

[17] van der Avoort J.A., Shirango H., Hoevenaars B. M.,Greffe J.M., de Hullu I.A.,de Wilde P.C. *et al.*: "Vulvar Squamous cell carcinoma is a multifocale disease following two separate and independent pathways". *Int. J. Gynecol. Pathol.,* 2006, *25,* 22.

[18] Hillemanns P., Wang X.: "Integration of HPV-16 and HPV-18 DNA in vulvar intraepitehelial neoplasia". *Gynecol. Oncol.,* 2006, *100,* 276.

[19] Lehtinen M., Koskela P., Jellum E., Bloigu A., Antilla T., Hallmans G., Luukkaala T. *et al.*: "Herpes simplex virus and risk of cervical cancer: a longitudinal, nested case-control study in the nordic countries". *Am. J. Epidemiol.*, 2002, *156,* 687.

[20] Tran-Thanh D., Provencher D., Koushik A., Duarte-Franco E., Kessous A., Drouin P. *et al.*: "Herpes simplex virus type II is not cofactor to human papillomavirus in cancer of the uterine cervix". *Am. J. Obstet. Gynecol.,* 2003, *188,* 129.

[21] Smith J.S., Herrero R., Bosetti C., Munoz N., Bosch F.X., Eluf-Neto J., Castellsouge X. *et al.*: "Herpes simplex virus-2 as a human papillomavirus cofactor in the etiology of invasive cervical cancer". *J. Natl. Cancer Inst.,* 2002, *94,* 1604.

[22] Costa S., Rotola A., Terzano P., Poggi M.G., Di Luca D., Aurelian L. *et al.*: "Search for herpes simplex virus 2 and human papillomavirus genetic expression in vulvar neoplasia". *J. Reprod. Med.,* 1990, *35,* 1108.

[23] Kwaśniewska A., Semczuk M., Kuźma D., Teresińska D., Goździcka-Józefiak A.: "Częstość występowania HPV i *Chlamydia trachomatis* w komórkach sromu kobiet asymptomatycznych". *Pol. Przeg. Gin. Pol.,* 2003, *3,* 139.

[24] Yang Y.Y., Koh W., Tsai 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.

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