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Clinical follow-up of women infected with human papillomavirus-16,
either alone or with other human papillomavirus types: identification
of different risk groups

THESE

préparée sous la direction du Docteur Stefan GERBER, Privat-docent et Maître
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par

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Objectifs

Evaluer l'impact clinique de femmes infectées par de multiples papillomavirus human (HPV) à haut risque dont le HPV 16 en comparaison de l'évolution de femmes infectées par du HPV 16 seul.

Méthode

169 femmes ont été classifiées en trois groupes, dépendant de leur profile HPV: HPV-16 seul, HPV-16 et un HPV de type bas risque, HPV-16 et un autre HPV à haut risque. Le HPV-DNA des frottis cervicaux a été analysé par polymerase chain reaction (PCR) et reverse line blot hybridization (RLBH). Toutes les femmes ont été suivies à la consultation de colposcopie pour une durée de 24 mois ou plus. La prise en charge s'est faite selon les recommandations de Bethesda.

Résultats

Les femmes infectées par du HPV 16 et un autre HPV à haut risque n'ont présenté aucun changement voire une progression de leur dysplasie en comparaison des femmes des autres groupes (RR: 1.39; 95%CI: 1.07 à 1.82; p value: 0.02 à 6 mois; RR: 2.10; 95%CI: 1.46 à 3.02; p value: <0.001 à 12 mois; RR: 1.82; 95%CI: 1.21 à 2.72; p value: 0.004 à 24 mois).

Conclusions

Les femmes présentant une co-infection par du HPV 16 ainsi qu'un autre HPV de haut risque voient leur risque d'évolution défavorable augmenter.

ONCOLOGY

Clinical follow-up of women infected with human papillomavirus-16, either alone or with other human papillomavirus types: identification of different risk groups

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OBJECTIVES: Evaluation of the clinical impact of multiple infections of the cervix by human papillomavirus, including human papillomavirus-16, compared with single human papillomavirus-16 infection.

STUDY DESIGN: One hundred sixty-nine women were classified in 3 categories depending on their human papillomavirus profile: human papillomavirus-16 only, human papillomavirus-16 and low-risk type(s), and human papillomavirus-16 and other high-risk type(s). Cervical brush samples were analyzed for human papillomavirus DNA by polymerase chain reaction and reverse line blot hybridization. All women were evaluated with colposcopy during 24 months or more. Management was according to the Bethesda recommendations.

RESULTS: Women infected with human papillomavirus-16 and other high-risk human papillomavirus type(s) presented more progression or no change in the grade of dysplasia, compared with women of the other groups (relative risk [RR], 1.39; 95% confidence interval [CI], 1.07-1.82; $P = .02$ at 6 months; RR, 2.10; 95% CI, 1.46-3.02; $P < .001$ at 12 months; RR, 1.82; 95% CI, 1.21-2.72; $P = .004$ at 24 months).

CONCLUSION: Coinfection of women with human papillomavirus-16 and other high-risk human papillomavirus type(s) increases the risk of unfavorable evolution.

Key words: cervical cancer, cervical dysplasia, human papillomavirus infection, human papillomavirus typing

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Cervical cancer is the second most frequent cancer in women worldwide and the principal cancer in most developing countries where 80% of the cases occur.^{1,2} Certain types of human

★ EDITORS' CHOICE ★

papillomaviruses (HPV) are now known to be the cause of this disease. HPV infection is a common sexually transmitted disease, which is spontaneously cleared in more than 70% of cases within 1 year.³⁻⁵ Women with a persistent infection have, however, a high risk for cervical cancer and its precursor lesions (cervical intraepithelial neoplasia [CIN]) to develop.^{6,7} Screening programs to identify CIN reduced significantly the mortality and morbidity of this disease.⁸⁻¹⁰ More than 100 HPV types have been identified to date, but only a subset is found to be associated with malignancy. HPV-16 and HPV-18, together with HPV-31, -33, -35, -39, -45, -51, -52, -58, -59, and others are detected in more than 95% of all cervical carcinomas.¹¹⁻¹³ Other anogenital HPV types are rarely associated with malignant tumors and have therefore been classified as "low-risk" (HPV-6, -11, -42, and others) or "intermediate-risk" types.¹⁴ Recently, a

possible association based on phylogenetic trees has been described between HPV-26, -53, and -66 and high-grade disease, though not necessarily cervical cancer.^{15,16}

Because women infected with high-risk HPV types are considered to be at a higher risk for the development of cervical cancer than those who are not infected with HPV or infected with low-risk HPV types, HPV screening is seen as an adjunct to cytologic diagnosis (Papanicolaou [PAP] test smear).¹⁷ Recently, it has been proposed that it may become the first step for cervical cancer screening.¹⁸ HPV testing allows also correct classification of women presenting with borderline PAP smears (atypical squamous cells of undetermined significance [ASC-US]).^{8,9,19}

A relatively large number of high-risk HPVs have been shown to persist as coinfections on the cervix, raising the possibility that oncogenic transformation and/or persistence may depend on the types of viruses that coexist in the

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cervix. Proportions of multiple HPV infections vary from 7% to 44%.²⁰⁻²⁵ HPV coinfection was found less frequently in cervical carcinoma than in normal cytology and in precancerous lesions,²⁶ consistent with the observation that cervical neoplasia is the result of a clonal expansion of a cell infected by a single HPV type.^{7,11} However, the outcome of the lesion may possibly have a relation with the profile of HPV genotypes.^{27,28} To test this hypothesis, we have compared the clinical course of women infected only with HPV-16 with that of women infected with HPV-16 and other HPV, either high or low risk.

MATERIALS AND METHODS

Study design and patients

We conducted this retrospective cohort study from 2000-2003, within the Department of Obstetrics and Gynecology, CHUV (University Hospital of Lausanne), Lausanne, Switzerland. This study was approved by the institutional Ethics Committee.

Inclusion criteria for women were: the identification of an HPV-16 infection on an abnormal Pap smear performed in liquid-based solution; a delay of less than 4 months before the first colposcopy; and a previous normal Pap smear within a year. A regular follow-up with colposcopy had to be documented. The classification, follow-up, and treatment in our colposcopy consultation is in accordance with the Bethesda recommendations. Exclusion criteria were pregnancy, HIV positive, or immunosuppressive treatment.

Cytology and histology were performed at the Institute of Pathology. Cervical cells were collected with a cytobrush and dispersed in a standard liquid solution. All histologic specimens were reviewed by an experienced pathologist.

Three groups were defined: group 1, women infected with HPV-16 only; group 2, women infected with HPV-16 and low-risk type(s); and group 3, women infected with HPV-16 and other high-risk HPV type(s). Undetermined-risk HPV types were considered as low-risk types and included in group 2.

HPV genotyping

HPV typing was performed systematically at inclusion and on subsequent examinations if surgery had been indicated. Typing was performed at the Institute of Microbiology as part of its routine diagnostic testing using accredited procedures (EN17025). Cells from 200 μ L liquid-based solution were collected and DNA was purified using the Magna Pure DNA isolation kit (kit no. 3003990; Roche, Rotkreuz, Switzerland) on the Magna LC robot (Roche), and eluted in 100 μ L elution buffer. HPV DNA was detected by polymerase chain reaction (PCR) and genotyped by reverse line blot hybridization (RLBH). PCR was performed with 5 μ L DNA in triplicate 50 μ L reactions using the PGMY primers according to Gravitt et al,²⁹ with slight modifications. HLA-DQA primers³⁰ were used as an internal standard to assess the quality of the DNA and absence of PCR inhibitors. After PCR, 5 μ L of each reaction was analyzed by gel electrophoresis and staining with ethidium bromide. Samples were considered informative if the HLA or the HPV DNA fragment could be detected. RLBH was performed on positive samples, essentially as described by Kaufhold et al,³¹ by using a panel of 31 HPV type-specific probes (high-risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, MM4 [type 82], and MM9 [type 73]; low-risk: 6, 11, 34, 40, 42, 44, 53, 54, 57, 70, and MM8 [type 84]; undetermined-risk: 26, 55, 66, and MM7 [type 83]). This procedure has been validated in the course of the first World Health Organization international collaborative study on detection of human papillomavirus DNA.³² To identify types not represented in the panel of probes, sequencing was performed on PCR-positive/hybridization-negative samples with sequencers from Applied Biosystems using the Big Dye Terminator chemistry (BDT v.1.1; Applied Biosystems, Foster City, CA) and PGMY11 primers. For the purpose of this study, those types that did not belong to the high-risk group defined previously were classified as low risk, despite recent publications that suggested a probable high-risk cytologic progres-

sion, although not oncogenic, associated with HPV-26, -53, and -66.^{15,16}

Follow-up

We recorded the following characteristics of included women: age, number of pregnancies, parity, smoking, and use of contraceptive methods.

All women were evaluated every 6 months by cytology and colposcopy, and directed tumor-biopsy specimen was performed when clinically indicated according to cytology results.

Histology was always performed to resolve ASC-US cases into histologic grades. When cytologic grades did not match the histology, the worse grade was used to classify the women. Those grades were used to assess evolution of the women.

When a treatment was indicated, laser vaporization or loop electrosurgical excision procedure (LEEP) was performed.

Evolution was described by 2 variables depending on the histology and/or the cytology. First, referred to as no-improvement, defined as *progression or no change* in the grade of dysplasia during the follow-up. Second, unfavorable evolution was defined as *progression* (referred to as progression) to a higher grade of dysplasia during the follow-up. All patients were monitored during 24 months or more with colposcopy, except those considered as cured. Complete recovery to normal state was defined as 3 consecutive normal cytology assessments.

Statistical analysis

Relative risks (RRs) and 95% confidence intervals (CIs) of no improvement and unfavorable evolution were calculated, comparing group 3 with groups 1 and 2, considered together as the reference group. Differences between proportions were tested using the χ^2 test. A *P* value less than .05 was considered as indicating statistical significance.

The potential confounding effect of treatment was evaluated in a stratified analysis (ie, women with and women without an intervention during follow-up). Adjusted RR was calculated using the Mantel-Haenszel method.

RESULTS

Characteristics of participants

From 2000-2003, 3010 women were followed at the colposcopy unit. According to exclusion criteria 826 women (27.4%) were excluded and 1221 (40.6%) did not fit the inclusion criteria, mainly because of an abnormal Pap smear in the previous year or a delay of more than 4 months before the first colposcopy. Nine hundred sixty-three women (32%) corresponding to inclusion/exclusion criteria have had an HPV typing. Of these 963 women, 741 were HPV-16 negative (77%) and 222 (23%) were infected with HPV-16. Fifty-three women were excluded because of incomplete follow-up.

The distribution of the resulting 169 cases within groups was as follows: 74 in group 1 (44%), 27 in group 2 (16%), and 68 in group 3 (40%). The median age was 27 years (range, 17-70 years), 71% had never been pregnant, 51% reported having smoked at least 20 cigarettes per day, and 56% reported having used oral contraception. There were no significant differences in the distribution of these factors between the 3 groups.

Distribution of HPV types and cervical dysplasia

Among the 74 women in group 1 at inclusion (time 0), 7 (9.5%) were classified as ASC-US, 45 (60.8%) as low-grade squamous intraepithelial lesion (L-SIL), and 22 (29.7%) as high-grade squamous intraepithelial lesion (H-SIL) (Table 1). For group 2 and group 3 they were, respectively, 1 of 27 (3.7%) and 13 of 68 (19.1%) ASC-US, 23 of 27 (85.2%) and 46 of 68 (67.7%) L-SIL, and 3 of 27 (11.1%) and 9 of 68 (13.2%) H-SIL. The evolution of all cases according to initial cytology is presented in Table 2. At time 0, there was no case of cervical cancer. Complete regression was observed in 26% of cases at 6 months of follow-up, 55.1% at 12 months, 82.2% at 24 months, and 95% at more than 24 months. After more than 24 months of follow-up, we observed the persistence of dysplasia in 9 women (3 in each group), despite the management.

TABLE 1

Distribution of HPV type and cytology classification at inclusion time

Variable	No. cases	ASC-US	L-SIL	H-SIL
Group 1 (HPV-16)	74 (44%)	7 (9.5%)	45 (60.8%)	22 (29.7%)
Group 2 (HPV-16 and low risk)	27 (16%)	1 (3.7%)	23 (85.2%)	3 (11.1%)
Group 3 (HPV-16 and high risk)	68 (40%)	13 (19.1%)	46 (67.7%)	9 (13.2%)
Total	169 (100%)	21 (12.4 %)	114 (67.5 %)	34 (20.1 %)

ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion.

Cottier. Risk-group identification for HPV-16 and other HPV types. Am J Obstet Gynecol 2009.

Evolution according to the HPV profile

The percentage of women showing no improvement was similar in group 1 and group 2 during each time interval (Figure 1 and Table 3). During the complete follow-up of 24 months, there was a persisting abnormality in 25.7% and 29.6% of women in group 1 and group 2, respectively. The risk of no improvement after 6 months was higher in group 3 compared with group 1 and group 2 (relative risk [RR], 1.39; 95% confidence interval [CI], 1.07-1.82; $P = .02$). This observation was confirmed at 12 months (RR, 2.10; 95% CI, 1.46-3.02; $P < .001$) and at 24 months (RR, 1.82; 95% CI, 1.21-2.72; $P = .004$).

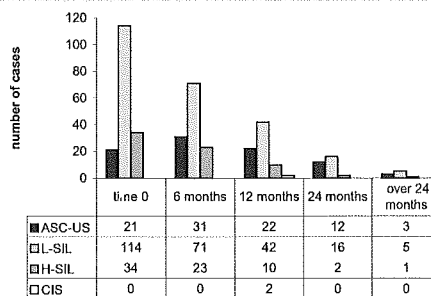
We observed a progression of the cervical lesion at 6 months in 16.2% of women in group 3, compared with 10.8% in group 1 and 11.1% in group 2 (Figure 2 and Table 4). The risk of progression at 6 months was higher, but not statistically significant, for women in

group 3, compared with group 1 and group 2 (RR, 1.49; 95% CI, 0.68-3.23; $P = .32$). This trend was confirmed at 12 months: 14.7% of women in group 3 had a progression of the lesion, compared with 6.8% and 3.7% in group 1 and group 2, respectively (RR, 2.48; 95% CI, 0.94-6.49; $P = .06$). This became statistically significant at 24 months (13.2% of women in group 3, compared with 4.1% and 3.7% in group 1 and group 2, respectively) (RR, 3.34; 95% CI, 1.07-10.42; $P = .03$). Evolution of cases of group 1 and group 2 were not statistically different during the complete follow-up with about only 4% of progression for both groups.

The interventions reduced the risk of no improvement (RR, 0.69; 95% CI, 0.46-1.04), but this was not statistically significant. We analyzed the effect of interventions performed during the follow-up on the relative risk of no improvement. The relative risk of group 3

TABLE 2

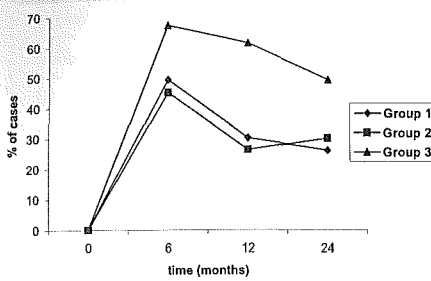
Distribution of cytologic diagnosis during follow-up



ASC-US, atypical squamous cells of undetermined significance; CIS, carcinoma in situ; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion.

Cottier. Risk-group identification for HPV-16 and other HPV types. Am J Obstet Gynecol 2009.

FIGURE 1
Percentage of women showing no improvement during the follow-up



Cottier. Risk-group identification for HPV-16 and other HPV types. *Am J Obstet Gynecol* 2009.

compared with group 1 and group 2 was similar in the subgroup of women with an intervention (RR, 2.24; 95% CI, 1.19-4.21) and in the subgroup of women without intervention (RR, 1.56; 95% CI, 0.93-2.62). The RR adjusted for intervention was 1.68 (95% CI, 1.24-2.78).

COMMENT

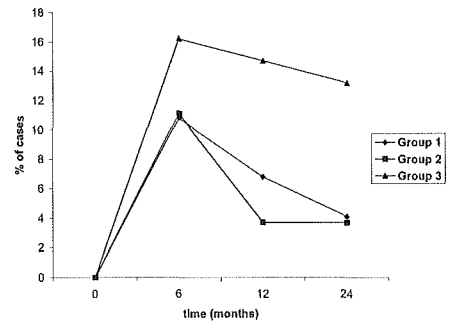
Our study shows that women infected with HPV-16 and another high-risk type (group 3) are at higher relative risk of unfavorable outcome compared with women infected either by HPV-16 only (group 1) or by HPV-16 and a low-risk type (group 2), consistent with the observations of Trottier et al.²⁸ HPV types of undetermined risk are rarely, if ever, detected in cervical cancer¹¹ and were thus included in the low-risk group for the statistical comparison. Classification of undetermined-risk viruses in the low-

risk category (group 2) was justified by their neutral statistical effect when they were included either in group 2 or in group 3 (not shown). Thus, we compared the clinical outcome of the group 3 women against the group 2 and group 1 women taken together as control.

At inclusion time, there was no identifiable group of patients especially at risk of cytologic progression when only the socioeconomical or risk factor characteristics other than HPV were taken into consideration. By definition, all study groups were considered high risk because of inclusion of HPV-16 in each. Yet, women infected with HPV-16 and another high-risk type (group 3) exhibited a worse clinical outcome compared with women infected by HPV-16 only (group 1) or by HPV-16 and a low-risk type (group 2). The only prognostic difference of the 3 patient groups was their initial profile of HPV infection, because they were all subjected to the same clinical management. Thus, group 3 women presented a higher risk of no improvement after 24 months and progression compared with women of the other 2 groups. At the present time, most gynecologic practices classify groups of HPV infections as high risk vs low risk, without identifying different subgroups. Diagnostic tests able to identify multiple infections should be used to take advantage of our observation. Such tests are now commercially available but need further validation and standardization for approval in clinical practice.³²

We were unable to analyze the individual contribution of each HPV type to the

FIGURE 2
Percentage of women with progression of the cervical lesion during follow-up



Cottier. Risk-group identification for HPV-16 and other HPV types. *Am J Obstet Gynecol* 2009.

clinical outcome, as the number of women coinfecting with each specific HPV was too small, and systematic HPV typing during the follow-up was not performed. We could not evaluate, in case of coinfections, the contribution of each type to cervical cancer progression. The worst outcome of group 3 compared with group 1 could be the consequence of a stronger way of high-risk types to overcome the natural mucosal defense through a synergistic effect mixed infection. Mixed infections with high-risk types may weaken the local immune host response, similar to the major immune escape described in many tumors, that targets the local innate immunity, through human α -defensin activity³³; this local immunity could be overridden by mixed infections with high-risk types.

The host immune cell mediated response is essential to reduce and to suppress the HPV infection. The interferon produced by the T cell and the natural killer cells leads to the regression of the HPV infection by antiviral, antiproliferative, and immunomodulatory activities. Although their synergy is not clearly established, mixed high-risk types could have a stronger negative impact on interferon production by decreasing Th1 response.^{34,35} We can only speculate that mixed infections with high-risk types (group 3) increase the chance of a successful clonal progression of singly infected cells. It will be necessary to develop a method to assess the immune

TABLE 3
Number and percentage of women showing no improvement during follow-up

Time	Start (n = 169)	6 mo (n = 169)	12 mo (n = 144)	24 mo (n = 78)
Group 1 (HPV-16)	74	36 (48.6%)	22 (29.7%)	19 (25.7%)
Group 2 (HPV-16 and low risk)	27	12 (44.4%)	7 (25.9%)	8 (29.6%)
Group 3 (HPV-16 and high risk)	68	45 (66.2%)	41 (60.3%)	33 (48.5%)
P value		.02	< .001	.004

HPV, human papillomavirus.

Cottier. Risk-group identification for HPV-16 and other HPV types. *Am J Obstet Gynecol* 2009.

TABLE 4
Number and percentage of women with progression during follow-up

Time	Start (n = 169)	6 mo (n = 169)	12 mo (n = 144)	24 mo (n = 78)
Group 1 (HPV-16)	74	8 (10.8%)	5 (6.8%)	3 (4.1%)
Group 2 (HPV-16 and low risk)	27	3 (11.1%)	1 (3.7%)	1 (3.7%)
Group 3 (HPV-16 and high risk)	68	11 (16.2%)	10 (14.7%)	9 (13.2%)
P value		.32	.06	.03

HPV, human papillomavirus.

Cottier. Risk-group identification for HPV-16 and other HPV types. *Am J Obstet Gynecol* 2009.

local mucosal response and to link it with HPV type, single or multiple, and cytology diagnosis (ongoing study). It appears that the immune response of the host plays a major role. This point of view is also very important in terms of vaccination strategies, prophylactic or therapeutic vaccine with multivalent HPV types, particularly considering the combination of HPV-16 and HPV-18, the 2 most common high-risk HPV types. It is not known whether subjects with multiple infections, including several cancer-associated HPV, would be protected by a vaccine not including all cancer-associated types. Interest in coinfections has increased in response to the possibility of vaccination. The host immune response appears to be mainly HPV type specific, but cross-protection could offer also a possible benefit, especially for women infected by multiple HPV types.^{36,37} For the present, commercial involvement essentially led to typization by risk group of HPV, and the future focuses on a promising multivalent HPV vaccine.³⁸

Our study supports the identification of a high-risk group of women, infected by multiple high-risk HPV types in addition to HPV-16. Further studies are needed to evaluate the effectiveness and cost of a specific follow-up for this group. In a therapeutic point of view, a prospective study would be needed to improve the management of this high-risk subgroup of women, perhaps with a combined approach of local immunomodulation and surgery. ■

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