Human papillomavirus infection in the female population of Antwerp, Belgium: prevalence in healthy women, women with premalignant lesions and cervical cancer

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Summary

Worldwide there is a strong relation between the presence of human papillomavirus (HPV) and the development of cervical cancer. This study investigated the prevalence and genotype of HPV in women with normal smears, women with premalignant lesions and women with cervical cancer in Antwerp, Belgium. Type-specific polymerase chain reaction (PCR) for HPV types 16 and 18 and general primer PCR (GP5+/6+) was performed on DNA extracted from paraffin-embedded tissue from women with lesions or fresh material from controls. HPV was detected in 11% of controls, 61% of women with atypia, 77% of women with CIN lesions and 88% of women with cervical carcinoma (X^2 trend, 273, $p<0.001$). The odds ratio for high-risk HPV types was 9.3 for atypia (95%Cl, 4.3-19.8), 33.6 for CIN lesions (95%Cl, 19.3-58.6) and 78.8 for cervical cancer (95%Cl, 39.2-158.3). In total, 19 different HPV genotypes were detected, including five low risk HPV types. Seven of the 14 high-risk HPV types were detected in cervical cancer patients. Based on our study it is suggested that a prophylactic vaccine based on a cocktail of a limited number of high-risk HPV types should be considered in order to protect most women from developing cervical cancer.

Key words: Cervical cancer, human papillomavirus, prevalence, PCR.

Introduction

Carcinoma of the uterine cervix is the second most common female malignancy worldwide, with the highest incidence rates observed in developing countries [1]. In contrast, in many developed countries the incidence and mortality of cervical cancer are falling [1]. Since the suggestion of a possible role of human papillomavirus (HPV) in the genesis of cervical cancer [2], it has been shown that infection with oncogenic HPV types, such as HPV types 16 and 18, is the most significant risk factor for the development of premalignant lesions of the cervix [3], which may eventually progress to cervical cancer. From a large study investigating invasive cervical cancer cases from 22 countries around the world it appeared that HPV type 16 predominates in squamous cell carcinomas, whereas HPV type 18 predominates in adenocarcinomas [4]. However, both ethnic and geographical variations in the prevalence of HPV types were demonstrated [4]. A recent extension on this study showed that HPV DNA was found in 99.7% of all evaluable cancers [5], suggesting that HPV-negative cervical carcinoma is extremely rare, if it exists at all. This strong relation between HPV and malignant disease has spurred interest in the development of both prophylactic and therapeutic vaccines. For an optimal introduction of vaccines it is essential to know the prevalence of HPV, not only in cervical cancer patients, but also in the healthy population. Here, we report the HPV prevalence in the female population in Antwerp, Belgium. Moreover, the study design chosen allowed us to assess the association between HPV exposure and cervical cancer by calculating the exposure odds ratios in cases and controls.

Materials and Methods

Patients and controls

Cervical cancer patients (N = 120, 78% squamous carcinoma, 12% adenocarcinoma, 7% adenosquamous carcinoma and 3% other carcinomas) and patients with varying degrees of dysplasia (N = 202) were selected from the records of the University Hospital Antwerp, Edegem, and the St. Augustinus Hospital Wilrijk, Belgium.

The control group (N = 286) consisted of women who presented at the Gynaecology Department of the University Hospital Antwerp for regular cytological cervical cancer screening. Selection criteria were ß-globin positivity and a normal Pap smear result. Controls provided a cervical scrape using a cytobrush. The brush was first used to prepare two glass slides for cytology and subsequently immersed in 3 ml ethanol in a 15 ml tube.

DNA isolation from tissue

DNA was extracted from 10 µm tissue sections by deparaffination with xylene and digestion with proteinase K as described by Wright and Manos [6]. Negative controls, consisting either of sections cut from paraffin blocks without tissue, or empty

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tubes processed in exactly the same way as the tissues, were introduced after every fifth sample to check for contamination.

DNA isolation from cells adhering to brushes

Afer vigorous vortexing of the tubes, the cells were pelleted and the brush removed. After phenol extraction and ethanol precipitation the DNA was resuspended in 100 µl TE (10 mM Tris 1 mM EDTA, pH 8) and stored at –20 °C until use. Negative controls, consisting of tubes without a brush, were introduced after every fifth sample to check for contamination.

HPV DNA analysis

Both in paraffin-embedded and in fresh material adequate DNA isolation was verified by a ß-globin polymerase chain reaction (PCR), using the PC03/PC04 primer set [7]. ß-Globin PCR positive paraffin-embedded material was first screened with type-specific (TS) PCR for HPV types 16 and 18 as previously described [8]. Cases negative in the TS PCR were screened with the GP5+/6+ PCR [9]. PCR products were analysed by agarose gel electrophoresis. GP5+/6+ PCR products were subsequently blotted and hybridised under low-stringent conditions with a cocktail probe consisting of HPV 6-, 11-, 16-, 18-, 31-, and 33- specific GP5+/6+ PCR products [10]. GP5+/6+ amplimers were purified using the Wizard PCR prep DNA isolation system (Prmega, Leiden, The Netherlands), and subsequently sequenced to determine the HPV type.

Fresh material from ß-globin positive controls was directly screened in the GP5+/6+ PCR. Positive samples were subjected to HPV 16 and HPV 18 TS PCR. GP5+/6+ positive, TS negative samples were sequenced to determine the HPV type.

Statistical analysis

The Chi squared test for trend was used to compare frequencies among the different study populations. Two times two tables were used to determine the odds ratio. A p-value smaller than 0.05 was considered statistically significant.

Results

The age distribution was very similar in controls and cervical intraepithelial neoplasia (CIN) patients, but women with atypia and cervical carcinoma patients were significantly older (Table 1).

All paraffin-embedded formaldehyde-fixed materials were initially screened for adequate DNA isolation by a ß-globin PCR generating an amplimer of approximately 100 base pairs. In four atypical lesions, four CIN lesions and five cases of cervical cancer a ß-globin amplimer could not be generated, even after 1 in 10 dilution of the target to overcome PCR inhibition. These samples were not further investigated.

Table 1. — *Age distribution of patient and control groups*

	N.	Mean age (SD)	Range
Controls	286	39.4 (13.60)	17-78
Atypia	43	45.2 (13.63)	26-81
Cervical Intraepithelial Neoplasia			
CIN I	58	36.3 (11.46)	19-77
CIN II	43	37.2 (10.19)	22-65
CIN III	58	36.9 (10.11)	20-63
Cervical cancer patients			
FIGO I	52	48.0 (12.99)	29-74
FIGO II	45	58.9 (15.17)	24-90
FIGO III	15	53.6 (14.79)	35-84
FIGO IV	8	62.7 (15.48)	40-87

FIGO - International Federation of Gynaecology and Obstetrics

The HPV presence and prevalence of genotypes in all populations is presented in Table 2. Overall, HPV was detected in 11% (31/286) of the controls, 62% (24/39) of the women with atypia, 77% (120/155) of the women with CIN lesions and 88% (101/115) of the women with cervical carcinoma (X^2 for linear trend = 273, $p < 0.001$). A linear trend was also found within the group of CIN lesions ($X^2 = 4.4$, $p < 0.05$), however, there was no linear trend with stage in the group of cervical cancer patients ($X^2 = 0.3$, p = NS). In total 19 different HPV genotypes were detected in this study, 11 genotypes in women with normal smears, 15 genotypes in CIN patients and seven genotypes in cervical cancer patients. Six (19%) women with a normal smear, one woman wih atypia (5%) and three women with CIN I lesions (7%) were infected with a low-risk HPV type (HPV types 6, 11, 40, 42, and 43). In one woman with a CIN III lesion a low-risk HPV type (HPV type 43) was detected, but this was a double-infection together with a high-risk HPV type. No low-risk HPV types were detected in women with CIN II lesions or in women with cervical cancer. Furthermore, in five women

Table 2. — *HPV prevalence and genotype in the study population*

	N	-ve	6	11	16	18	31	33	35	39	40	42	43	45	51	52	56	58	59	66	68	x
Control	286	255	4	–	8	4	–	1	–	2	–	1	1	2	1	–	–	1	1	–	–	5
Atypia	39	15	–	–	10	7	–	–	–	–	1	–	–	–	–	–	–	1	–	–	–	5
CIN I	58	18	–	1	14	11	1	–	–	–	1	–	1	3	2	1	3	–	–	1	1	–
CIN II	42	9	–	–	23	1	–	2	1	–	–	–	–	–	1	2	–	1	–	1	–	1
CIN III[1]	55	8	–	–	32	5	–	4	1	–	–	–	1	–	–	–	2	5	–	–	1	–
Total CIN[1]	155	35	–	1	69	17	1	6	2	–	1	–	2	3	3	3	5	6	–	2	2	1
FIGO I[2]	49	8	–	–	32	7	1	–	–	–	–	–	–	1	–	–	–	–	–	1	–	–
FIGO II	45	2	–	–	32	5	2	2	–	–	–	–	–	1	–	–	–	–	–	–	–	1
FIGO III	14	2	–	–	8	3	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–
FIGO IV	7	2	–	–	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CaCx[2]	115	14	–	–	77	15	3	2	–	–	–	–	–	2	–	–	1	–	–	1	–	1

[1] Including four double-infections; once 16/43, once 33/35 and twice 33/58.

[2] Including one 16/18 double-infection.

with a normal smear, five cases of atypia, one case of CIN and five cases of cervical cancer the HPV type(s) present could not be determined, due to multiple infection, dirty template or lack of material, rather than the presence of new, as yet unsequenced HPV types. For calculation of odds ratios, the unknown HPVs were divided over low-risk and high-risk types in the same ratio as the HPVs that could be determined. The odds ratio as well as the confidence interval is presented in Table 3.

Table 3. — *Odds ratios for atypia, CIN and cervical cancer*[1]

	N.	Neg.	LR	HR	OR[2]	C.I.
Controls	286	255	7	24	1	(referent)
Atypia	39	15	1	23	9.3	4.3-19.8
CIN	155	35	3	117[3]	33.6	19.3-58.6
CaCx	115	14	0	101	78.8	39.2-158.3

LR, low-risk (i.e. 6, 11, 40, 42 and 43), HR, high-risk (i.e. 16, 18, 31, 33, 35, 39, 51, 52, 56, 58, 59, 66 and 68), OR, odds ratio, C.I., confidence interval, CaCx, cervical cancer

[1] Subjects scored as HPV type x were divided over HR and LR in ratios found

[2] The OR was calculated for HR versus (LR or HPV negative)

[3] One subject had a LR/HR double-infection and was scored as HR

Discussion

This study was performed to investigate the prevalence and genotype of HPV present in cervical specimens from invasive cancer, pre-invasive disease and normal subjects in a population of women living in Antwerp, Belgium. Previous studies performed in Belgium were limited to the detection of HPV types 16, 18 and 33 [11, 12].

The prevalence of HPV in women with normal smears was 10.8%, which is in agreement with other studies in Western European countries: 9.6% in The Netherlands [13], 10% in Sweden [14], and 15.3% in Denmark [3], but much lower than the prevalence in a study in the United States, where 23.5% of the controls were HPV positive [15]. Differences in ethnic distribution within the two groups may play a role. The prevalence of high-risk HPV types 16 and 18 in controls in The Netherlands (3.8%) [13] is also comparable to the prevalence in our study (4.2%). The mean age in our control group is somewhat higher than in comparable studies in other Western European countries [3, 13, 14]. There are several explanations for this phenomenon. Firstly, there is no nationwide population-based cervical cancer screening in Belgium and women are free to continue screening after the age of 65, at which point population-based screening is normally stopped. Secondly, in Belgium women may choose to have their smears taken by a gynaecologist or a general practitioner. It has been shown that older women prefer to go to a gynaecologist [16]. Since our control group is derived from the Department of Gynaecology at the University Hospital Antwerp, the mean age of these women is somewhat higher than the mean age of the entire screening population. We are currently investigating whether there is a difference in the prevalence of HPV in both populations (gynaecologists versus general practitioners).

Although the diagnosis "atypia" covers a broad spectrum of minimal abnormalities, including atypical squamous cells of unknown significance (ASCUS), which does not qualify as a cervical intraepithelial lesion, this study confirms that HPV is causally involved in its development, since an odds ratio of 9.3 was found. A relative risk of 9.3 was also found in a prospective study in Denmark [17]. Indeed, recently it has been shown that for women with a diagnosis of ASCUS, HPV DNA testing can help identify those women with underlying high-grade neoplasias [18].

In women with CIN lesions the overall prevalence was 77.4%. The prevalence of high-risk types was 64% in CIN I, 76% in CIN II and 85% in CIN III, which is slightly different from the prevalence found in a group of 114 German women with CIN lesions [19] (CIN I, 47%; CIN II, 77%; CIN III, 97%). Furthermore, in the German study HPV type 39 was detected, which was only found in women with normal smears in Belgium. On the other hand, in the present study HPV types 35, 52 and 68 were detected, which did not occur in the German study. The odds ratio of 33.6 obtained when comparing high-risk HPV positivity in CIN patients and controls is even higher than in a Danish study in which it was concluded that HPV infection is by far the most significant risk factor for the development of intraepithelial lesions [3]. However, since our study is a retrospective study based on pathology findings only, it was impossible to investigate the role of other potential risk factors such as genetic predisposition and smoking habits.

A recent extension on the worldwide study of HPV in cervical carcinomas has shown that HPV DNA was detectable in 99.7% of all adequate samples [5]. To obtain this high percentage of HPV positives, three different PCR assays were used, targeting three different open reading frames, E7, L1 and E1. In this study a prevalence of HPV in cervical cancer patients of 88% was found. This may in part be due to the use of PCR assays directed against only two different open reading frames, of which the E7 PCR only specifically detected HPV 16 and HPV 18, admittedly the two most commonly detected HPV types in cervical carcinomas. Furthermore, verification of DNA isolation was done by a beta-globin PCR generating an amplimer of 100 basepairs, whereas the GP5+/6+ PCR generates an amplimer of 150 basepairs. Therefore, it cannot be excluded that some cases are positive for beta-globin but are not suitable for GP5+/6+ PCR. However, since type-specific PCR is also performed for HPV types 16 and 18, and this PCR generates and amplimer of approximately 100 basepairs, this would only be the case for women with an HPV infection other than 16 and 18, which is a minority of the cases.

The prevalence of HPV DNA in this study compares well with a previous study on formaldehyde-fixed material in The Netherlands [8]. Notwithstanding the lower sensitivity to detect HPV DNA in formaldehyde-fixed material compared to fresh tissue specimens, an odds ratio of 78.8 was found when the prevalence of high risk HPV in cervical cancer cases was compared with con-

trols. This compares favourably with a recent study performed in Morocco (OR = 61.6) [20], but is somewhat lower than the OR of 119 found in Thailand [21] and the Philippines (OR = 156) [22].

With respect to the development of prophylactic and therapeutic vaccines it can be concluded that the main genotypes present in high grade lesions including cancer are HPV types 16 and 18, as previously reported by many others. Some other genotypes are sporadically encountered in cervical carcinomas, such as HPV types 31, 33, 45, 56 and 66. However, some of the HPV types which are claimed to be high-risk, including HPV types 39, 51 and 59, were only detected in women with normal smears and women with low-grade lesions. HPV types 39 and 59 are frequently encountered in cervical carcinomas in Central and South America [4]. We showed that they are also present in the Belgian population, but there these types do not seem able to progress to cervical cancer or even high-grade lesions. This difference in oncogenicity may be related to differences in HLA composition in the two populations, and warrants further study. Finally, HPV type 18 is present relatively frequently in atypia and CIN I lesions, which is in agreement with other studies [23, 24]. These authors suggest that HPV 18 should consequently be ranked as a low-risk, rather than high-risk oncogenic HPV type. However, in our study, HPV 18 was also present in 9% and 13% of CIN III lesions and invasive tumours, respectively, indicating its high-risk oncogenicity. We are currently investigating whether intratype variation is involved in this low-risk versus high-risk discrepancy.

Conclusion

This study confirmed the relation of HPV with cervical cancer as well as premalignant lesions of the cervix. The number of different HPV types present in invasive tumours seems small, which would render it feasible to develop a prophylactic vaccine based on a cocktail of a limited number of HPV types, which should be able to protect most women from developing cervical cancer.

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