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RISK FOR CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE 3 OR WORSE IN RELATION TO SMOKING AMONG WOMEN WITH PERSISTENT HUMAN PAPILLOMAVIRUS INFECTION

Kirsten Egebjerg Jensen[1], Sven Schmiedel[1], Kirsten Frederiksen[1], Bodil Norrild[2], Thomas Iftner[3], Susanne K. Kjær[1,4]

[1] Danish Cancer Society Research Center, Strandboulevarden 49, 2100 Copenhagen O, Denmark

[2] Department of Cellular and Molecular Medicine, University of Copenhagen, Blegdamsvej 3B, 2200 Copenhagen N, Denmark

[3] University Hospital of Tübingen, Section Experimental Virology, Elfriede-Aulhorn-Strasse 6, 72076 Tübingen, Germany

[4] Gynecologic Clinic, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, 2100 Copenhagen O, Denmark

Corresponding author:

Susanne K. Kjær, Unit of Virus, Lifestyle and Genes, Danish Cancer Society Research Center, Strandboulevarden 49, DK-2100 Copenhagen O, Denmark

Phone: +45 35 25 76 63, Fax: +45 35 27 18 11, E-mail: susanne@cancer.dk

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ABSTRACT

Background: Smoking has been associated with cervical cancer. We examined whether smoking increases the risk for high-grade cervical lesions in women with high-risk human papillomavirus (HPV) infection.

Methods: In a population-based cohort study, 8,656 women underwent a structured interview, and subsequently cervical cells were obtained for HPV DNA testing. Women with high-risk HPV infection and no prevalent cervical disease at baseline (n=1,353) were followed through the Pathology Data Bank for cervical lesions for up to 13 years. Separate analyses of women with persistent high-risk HPV infection were also conducted. Hazard ratios (HRs) for a diagnosis of cervical intraepithelial neoplasia grade 3 or worse/high-grade squamous intraepithelial lesions or worse (CIN3+) and the corresponding 95% confidence intervals (CIs) were calculated in the 2 groups.

Results: Among high-risk HPV positive women an increased risk for CIN3+ was associated with long-term smoking (≥10 years) and heavy smoking (≥20 cigarettes/day). In the subgroup of women with persistent HPV infection heavy smoking was also associated with a statistically significantly higher risk for CIN3+ than never smoking (HR, 1.85; 95% CI, 1.05–3.22, adjusted for length of schooling, parity and HPV type at baseline). The average number of cervical cytology screening tests per year during follow-up did not explain the differences in risk in relation to smoking (p=0.4).

Conclusions: Smoking is associated with an increased risk for subsequent high-grade cervical lesions in women with persistent high-risk HPV infection.

Impact: Our study adds to the understanding of the role of smoking in the natural history of HPV and cervical carcinogenesis.

ABBREVIATIONS

CI: confidence interval

CIN3: cervical intraepithelial neoplasia grade 3

HC2: hybrid capture 2

HPV: human papillomavirus

HR: hazard ratio

HSIL: high-grade squamous intraepithelial lesions

INTRODUCTION

Cervical infection with high-risk human papillomavirus (HPV) is considered to be a necessary cause of cervical cancer (1). A single positive test for high-risk HPV (2;3) and, even more convincingly, a persistent infection with high-risk HPV are highly predictive of cervical intraepithelial neoplasia grade 3 (CIN3) or worse (3;4). Cervical HPV infection is not, however, sufficient for developing cervical cancer, and other factors, such as viral characteristics, environmental factors and host factors, may also play a role (5). Smoking (6;7) has been associated with cervical cancer and its precursor lesions, yet it is unclear whether smoking works indirectly by increasing the risk for HPV acquisition or HPV persistence or whether smoking is a HPV cofactor, which contributes to the risk for high-grade cervical disease in addition to HPV.

The association between smoking and high-grade cervical disease has been studied mainly in case-control studies (6–11) and less often in prospective cohort studies (12–14) and the results were based on a single measure of HPV, which captures both transient and persistent HPV infections. As progression of cervical disease depends on the presence of persistent high-risk HPV infection (15) HPV cofactors should be studied in a cohort of women with persistent infections, as determined by consecutive cervical measurements (5). To our knowledge the present study is the first long-term prospective study examining the risk of subsequent development of high-grade cervical disease among women with persistent high-risk HPV infection in relation to smoking. We reported previously on the short-term risk for a cytological diagnosis of high-grade squamous intraepithelial lesions (HSIL) among HPV-positive (single measurement) women in this cohort in relation to smoking (16).

The aim of the study reported here was to assess whether smoking plays a role in the subsequent development of high-grade cervical disease. This was studied in an overall study population of women with high-risk HPV infection and no prevalent cervical disease at

enrollment and in a subgroup of women with persistent HPV infection. The women were followed for up to 13 years.

MATERIALS AND METHODS

The study population consisted of women participating in a population-based prospective cohort study of the natural history of HPV in Denmark (17). Every citizen of Denmark has a unique personal identification number, which is registered in the computerized Civil Registration System and comprises information about sex and date of birth. This number is used throughout society, including the public administration and the health care system, and it enables researchers to conduct representative population-based studies and ensures correct linkages between registries in Denmark. We used the Civil Registration System to identify a random sample of women aged 20–29 years living in Copenhagen (n=17,949) and invited them to participate in the study. Women who had moved out of the study area before contact (n=1,604) were ineligible for the study. Between May 1991 and January 1993, 11,088 women were included in the study. Approximately 2 years later, the women were invited to participate in a second examination (October 1993–January 1995) in the same order as they were included originally; and 8,656 women participated (participation rate, 78%). At each visit, the women underwent a gynecologic examination with a Pap smear and cervical swabs were obtained and stored at -80°C for subsequent HPV DNA detection (17). The women were interviewed by trained female nurses using a structured questionnaire containing questions about sociodemographic factors, smoking behavior, contraceptive use, reproductive factors, sexual behavior and history of sexually transmitted infections. All participants provided written informed consent before entering the study. The study was approved by the national Scientific Ethical Committee and the national Data Protection Board.

HPV DNA testing

In all cervical samples, HPV DNA was detected by Hybrid Capture 2 (HC2; Qiagen-Hilden, Germany) and in those samples which were positive with the HC2 assay a polymerase chain reaction-based method was used for genotyping (17). The cut-off of 1.0 pg/ml recommended by the United States Food and Drug Administration was used for the HC2 assay, and only the high-risk probe that detects at least 13 high-risk types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) was used (18). Genotyping was performed with the LiPAv2 test (Innogenetics, Inc., Ghent, Belgium) (19), which allows identification of 24 HPV types of which we were interested in the high-risk types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68).

Follow-up

The Pathology Data Bank is a nationwide registry containing information on all cervical cytological and histological examinations (biopsies, cones, and hysterectomies; normal as well as abnormal diagnoses). The individual personal identification number was used as the key identifier of each woman in the study population to obtain information about cervical cytology and histology from the Pathology Data Bank during follow-up. Most abnormal cervical diagnoses were reported as atypia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma *in situ*, or cancer. The cytological diagnoses severe dysplasia, carcinoma *in situ*, and cancer were categorized as HSIL or worse, and the histological diagnoses of these lesions were translated into the CIN nomenclature as CIN3 or worse.

The study population used for the study reported here comprised women who participated in both examinations (n=8,656), as we wanted to study cervical outcomes subsequent to HPV DNA results from the 2 examinations. The women were followed from baseline, defined as the date of the second individual examination, until March 6, 2007. The women were followed for incident high-grade cervical disease i.e. a histological diagnosis of CIN3 or worse and/or a cytological diagnosis of HSIL or worse.

We restricted the analysis to the 1,435 women who had high-risk HPV infection at baseline (defined as a positive HC2 test with the high-risk probe at the second examination). We then excluded 21 women with an inadequate baseline smear and 35 women who had no cervical examinations during follow-up. Finally, we excluded 26 women with prevalent disease at baseline (moderate dysplasia or worse) or 1 year prior to baseline, resulting in 1,353 high-risk HPV positive women at baseline in the overall study population.

In addition to the overall high-risk HPV positive study population defined by a single HPV measurement, we used information from both cervical examinations to define a subgroup of women with persistent HPV infection. Women who were high-risk HPV positive at baseline and positive for the same high-risk HPV type (identified by the LiPAv2 test) at baseline and 2 years earlier (i.e. at the first examination) were defined as having persistent infection (n=312).

In the overall study population (n=1,353), a group of 241 women developed high-grade cervical disease during follow-up. The majority (n=187) had CIN3 or worse and 54 women had a diagnosis of HSIL or worse without histological confirmation. In the following this combined group is called CIN3+.

At baseline, i.e., the second interview, we obtained information about smoking status (never, former, current), age at smoking initiation, smoking duration and smoking intensity (the average number of cigarettes smoked per day when smoking the most).

Statistical analysis

Associations between the risk of developing CIN3+ and smoking were estimated using an accelerated failure time model, taking into account that the exact date of development of the cervical abnormality was unknown (interval-censored response variable). A Weibull distribution was used for the failure time, which fitted the data significantly better than the

more parsimonious exponential model (p<0.001) and as well as the more complex generalized gamma model (p=0.34). Hazard ratios (HRs) and their corresponding 95% confidence intervals (CIs) were calculated for CIN3+ in relation to smoking in the study population of all high-risk HPV positive women and in the subgroup of women with persistent HPV infection at baseline. When we restricted our analysis to diagnoses based only on histology (CIN3 or worse) we found similar risk patterns with wider CIs. Thus we included both cytological and histological high-grade cervical lesions in the final model. The proc lifereg procedure of the SAS software Version 9.1 was used for analysis.

The HRs were adjusted for length of schooling ($\leq$9 years, 10–11 years, $\geq$12 years) and ever given birth (yes/no). We also adjusted the final model for baseline HPV type (HPV16, non-16 high-risk HPV), as this is strongly associated with disease progression (2–4). Initially, we also adjusted for age at baseline but as this did not affect the overall results, it was not included in the final analysis. Young age at first intercourse, lifetime number of sexual partners, and use of oral contraceptives were also initially considered as potential confounders, but, as none of these variables were significantly associated with the outcome and did not change the overall estimates of the other variables, they were not included in the final models.

Finally, we investigated whether the average number of cervical cytology examinations per year in the 13-year follow-up period was associated with smoking status (never, former current) among the women with no abnormal cervical examinations registered during follow-up using F-test.

RESULTS

The age distribution of the overall study population of high-risk HPV positive women was even, 30.0% being 22–24 years old, 33.2% being 25–27 years old and 36.8% being 28–32 years old. Most women had 12 or more years of schooling (68.3%); 43.9% reported that they

were current smokers at baseline; 16.3% had given birth; and 54.0% reported fewer than 10 lifetime sex partners (data not shown).

During follow-up, CIN3+ was diagnosed in 241 women (17.8%) in the overall study population. When this study population was restricted to women with a persistent HPV infection at baseline, CIN3+ developed in 30.8% (96/312) of the women. The average number of cervical cytology examinations per year from entrance until end of study among women who had no abnormal cervical diagnoses during follow-up was not statistically significantly different by smoking status (current smoking, 0.30; former smoking, 0.29; never smoking, 0.29; p=0.4) (data not shown).

Table 1 presents the risk for a subsequent diagnosis of CIN3+ according to smoking in the overall study population of women who tested positive for high-risk HPV at baseline. Women who had ever smoked had an increased risk for CIN3+ (HR_{unadj}, 1.36; 95% CI, 1.03–1.80). After adjustment for length of schooling, ever given birth, and HPV type, the risk estimate was increased at borderline statistical significance (HR, 1.32; 95% CI, 1.00–1.76). When women who had ever smoked were divided into former smokers and current smokers, only those who were current smokers were significantly more likely to have a subsequent CIN3+ in the fully adjusted model (HR, 1.39; 95% CI, 1.03–1.87). Women who had smoked for 10 or more years (HR, 1.45; 95% CI, 1.04–2.02) had a significantly greater likelihood of CIN3+ than those who had never smoked. Heavy smoking ($\geq$20 cigarettes per day) also significantly increased the risk (HR, 1.51; 95% CI, 1.06–2.16). Women who were 15–18 years old when they began smoking had a significantly higher risk for CIN3+ than those who had never smoked (HR, 1.47; 95% CI, 1.08–2.00).

The HRs for a diagnosis of CIN3+ in relation to smoking in the subgroup of women with persistent HPV infection are shown in Table 2. Women who smoked 20 or more cigarettes per day were significantly more likely to be diagnosed with CIN3+ than those who never

smoked (HR, 1.84; 95% CI, 1.05–3.22). Increased risks were also observed with current smoking and longer smoking duration, however, they did not reach statistical significance.

DISCUSSION

It has previously been found that persistent high-risk HPV positivity significantly increases the risk of subsequent CIN3 or worse in prospective studies (3–4). Our study is the first longitudinal study of HPV cofactors exploring the subsequent risk of high-grade cervical disease given HPV persistence. Among women with persistent high-risk HPV infection heavy smoking significantly increased the risk of subsequent development of CIN3+. Women who were high-risk HPV positive based on a single HPV measurement had also an increased risk for CIN3+ in relation to smoking.

Tobacco smoke, a well-known carcinogen, is believed to influence the natural history of cervical cancer through several pathways in a complex manner (20). Compounds and metabolic products from smoke have been found in cervical tissue (21) and, e.g., benzo[*a*]pyrene, a major carcinogenic constituent of cigarette smoke, has been shown to increase HPV viral titers and genome copies in cervical cells, which potentially could enhance carcinogenesis (22). Furthermore, tobacco smoke alters various aspects of the immune function (23).

Previous studies of HPV positive women based on a single HPV measurement found that ever smokers and current smokers had an increased risk for high-grade cervical disease (7–14). In addition, increased risks have been observed in relation to heavy smoking (8–10;12;13) and some studies also observed an association with long-term smoking (8;13) but not all (9). A large multi-center case-control study did not observe a dose-response relation with duration of smoking, the number of cigarettes smoked per day or age at smoking initiation (7). In our study in the analysis of high-risk HPV positive women (single HPV

measurement) we also found that ever smoking and current smoking was statistically significantly associated with an increased risk for CIN3+ and in particular we observed elevated risks associated with heavy smoking and long-term smoking. When analyzing amount of smoking and duration of smoking separately among currently smoking women and among women who were former smokers, we observed similar patterns as observed for ever smokers, however the associations were only statistically significant for current smokers (data not shown).

Since HPV16 conveys a higher risk for high-grade cervical disease than other high-risk HPV types it could be hypothesized that smoking influenced the risk of CIN3+ differently dependent on HPV type. However, when we restricted the high-risk HPV positive women to respectively HPV16 positive women or to women positive for non-16 high-risk HPV types, we found similar results (data not shown). Previous studies also observed the same association with smoking when restricting the analysis to HPV16 positive women (11;13).

In our study the proportion of women who developed CIN3+ was higher among women with persistent HPV infection (30.8%) than in the overall study population of high-risk HPV positive women based on a single HPV measurement (17.8%). In studies based on a single HPV measurement associations between smoking and high-grade cervical lesions could theoretically be caused by an association between smoking and the risk of persistence. By using a study population of women with a persistent HPV infection this is not likely to be the explanation. When we analyzed the risk of CIN3+ among women with persistent high-risk HPV infection, we found that women who were heavy smokers had a significantly higher risk for CIN3+ than never smoking women. This indicates that in addition to the strong effect of the characteristic of the HPV infection (persistence), smoking is related to an increased risk for progression.

It has been suggested that smoking status in relation to patterns of participation in cervical cancer screening could explain the increased risks for cervical cancer associated with smoking (13;20). A study in Denmark found an association between attendance at a cervical cancer screening program and smoking status (24), however we found no difference in the average yearly number of cervical cytological tests during follow-up by smoking status (p=0.4); therefore, we do not find it plausible that different screening patterns could explain our findings.

Our study has several strengths. We were able to identify a cohort of women with persistent HPV infection and examine the subsequent for risk of CIN3+ among these women. In contrast previous studies used a single measure of HPV positivity and were therefore not able to differentiate between transient and persistent infections. Our approach implied that the observed risk of CIN3+ in relation to smoking could not be entirely explained by smoking having increased the likelihood of HPV persistence. Persistence was defined by detection of the same high-risk HPV genotype 2 years apart. This implies that the test interval is longer than the average clearance period for high-risk infections (25); however, this might also in theory capture some re-infections with the same genotype. To define HPV persistence by a wide test interval, such as 12 months or more, has shown to be a good predictor of subsequent progression (26). A 2-year test interval has also shown to be a good predictor in relation to HPV type-specific progression rates (3). An additional strength was that the participants originated from the general population of 20–29-year old women. Together with a relatively high participation rate it increases our ability to extrapolate our results to the general population in this age group. The study cohort was followed up for incident high-grade cervical disease over a long period (up to 13 years), and the follow up data originated from the nationwide Pathology Data Bank, which is known to be of high quality and virtually complete regarding cervical examinations and diagnoses. Furthermore, because of the existence of unique personal identification numbers, which are registered in the computerized Civil Registration System, we could follow-up the women with virtually no loss

to follow-up. To ensure a truly prospective design, we excluded women with prevalent disease at baseline and 1 year before baseline. Finally, we were able to adjust for several potential confounding factors.

The study also has some potential limitations. We did not have sufficient statistical power to analyze HPV cofactors in relation to cervical cancer and therefore studied the immediate precursor lesions. Furthermore, the group of women with persistent high-risk HPV infection was small, which might explain why there were only a few statistically significantly increased risk estimates. The risk pattern in women with persistent high-risk HPV infection should be explored on a larger scale. Our study design allowed us to conclude that the high-grade cervical disease developed subsequent to both HPV infection and smoking initiation. However, we had no information on the timing of cervical HPV infection relative to that of smoking initiation and whether this is important for cervical disease progression and we cannot make conclusions about the timing of smoking and HPV infection in relation to the subsequent risk of CIN3+. To understand this aspect in detail, further studies are needed. It is a limitation of our study that there was no centralized review of cytology and histology data. In our study as well as in other studies which rely on self-reported smoking habits it cannot be excluded that information about smoking is subject to some degree of underreporting which potentially could lead to a conservative estimate of the association.

In summary, we found that smoking is an HPV cofactor for cervical disease progression. Especially women with persistent high-risk HPV infection who are heavy smokers had a significantly increased risk for high-grade cervical disease. This study is the first to show a contribution of smoking in cervical carcinogenesis given persistent HPV infection.

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Table 1.

	cases/ non-cases	HR	HR[a]	HR[b]	(95% CI)
Smoking					
Never	71/409	1	1	1	
Ever	170/701	**1.36**	1.30	**1.32**	**(1.00–1.76)**
Smoking status[c]					
Former	46/231	1.14	1.12	1.20	(0.83–1.74)
Current	124/470	**1.47**	**1.39**	**1.39**	**(1.03–1.87)**
Smoking duration[c]					
≤4 years	22/106	1.20	1.19	1.25	(0.77–2.01)
5–9 years	69/305	1.27	1.24	1.25	(0.89–1.74)
≥10 years	79/290	**1.52**	**1.40**	**1.45**	**(1.04–2.02)**
Smoking intensity[c]					
1–9 cigarettes/day	38/187	1.16	1.12	1.15	(0.78–1.71)
10–19 cigarettes/day	74/301	1.37	1.31	1.33	(0.96–1.85)
≥20 cigarettes/day	58/211	**1.55**	**1.46**	**1.51**	**(1.06–2.16)**
Smoking initiation[c]					
≤14 years old	45/203	1.28	1.14	1.13	(0.77–1.68)
15–18 years old	99/360	**1.50**	**1.44**	**1.47**	**(1.08–2.00)**
≥19 years old	26/137	1.12	1.11	1.17	(0.75–1.84)
Age at baseline					
22–24 years old	67/338	1	1	1	
25–27 years old	80/369	1.10	1.08	1.10	(0.80–1.53)
28–32 years old	94/404	1.17	1.07	1.11	(0.80–1.54)
HPV type at baseline					
High-risk HPV non-16	138/899	1	1		
HPV16	103/213	**2.68**	**2.70**		

[a] Adjusted for ever given birth and length of schooling
[b] Adjusted for ever given birth, length of schooling and HPV type
[c] Never smoking is the reference category
Bold: p<0.05

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Table 2.

	cases/ non-case	HR	HR[a]	HR[b]	(95% CI)
Smoking					
Never	28/84	1	1	1	–
Ever	68/132	1.47	1.42	1.35	(0.86–2.12)
Smoking status[c]					
Former	20/46	1.31	1.23	1.20	(0.67–2.13)
Current	48/86	1.54	1.52	1.45	(0.89–1.45)
Smoking duration[c]					
≤4 years	9/16	1.54	1.43	1.27	(0.60–2.71)
5–9 years	27/56	1.40	1.39	1.38	(0.80–2.35)
≥10 years	32/60	1.51	1.43	1.36	(0.80–2.31)
Smoking intensity[c]					
1–9 cigarettes/day	14/35	1.19	1.22	1.18	(0.62–2.25)
10–19 cigarettes/day	29/59	1.41	1.27	1.19	(0.70–2.02)
≥20 cigarettes/day	25/38	**1.78**	**1.86**	**1.84**	**(1.05–3.22)**
Smoking initiation[c]					
≤14 years old	23/36	**1.80**	1.73	1.53	(0.86–2.75)
15–18 years old	38/80	1.33	1.29	1.26	(0.77–2.06)
≥19 years old	7/16	1.41	1.48	1.48	(0.64–3.43)
Age at baseline					
22–24 years old	26/69	1	1	1	–
25–27 years old	34/73	1.24	1.16	1.18	(0.70–1.98)
28–32 years old	36/74	1.27	1.13	1.18	(0.70–2.00)
HPV type at baseline					
High-risk HPV non-16	44/163	1	1		
HPV16	52/53	**2.69**	**2.50**		

[a] Adjusted for ever given birth and length of schooling
[b] Adjusted for ever given birth, length of schooling and HPV type
[c] Never smoking is the reference category
Bold: p<0.05

LEGENDS FOR TABLES

Table 1. HRs for a diagnosis of CIN3+ according to smoking among high-risk HPV positive women (n=1,353)

Table 2. HRs for a diagnosis of CIN3+ according to smoking among women with persistent high-risk HPV infection (n=312)

Cancer Epidemiology, Biomarkers & Prevention

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Risk for cervical intraepithelial neoplasia grade 3 or worse in relation to smoking among women with persistent human papillomavirus infection

Kirsten Egebjerg Jensen, Sven Schmiedel, Kirsten Frederiksen, et al.

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