Human Papilloma Virus Tests of Normal Cervical Smears Collected Prior to the Development of Squamous Carcinoma: A Pilot Study

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Sir,

Following the introduction of organized cytological screening in Sweden in the late 1960s, the incidence of cervical carcinoma decreased considerably. The estimated benefit of screening is a reduction in the number of cases of cervical cancer by at least 50% (1).

At present, most women diagnosed with cervical cancer have chosen not to participate in the screening. Approximately 25% of the remaining women with cervical cancer have attended screening, but repeated cervical smears have been considered normal (2–3).

When cytological screening was introduced, the pathogenesis of cervical cancer was unclear. Since then, it has become evident that a persistent oncogenic human papilloma virus (HPV) infection is the most prominent factor for tumour transformation (4). Accordingly, HPV analysis has been suggested as a complement, or even an alternative, to cytological screening. In comparison with cytology, HPV testing is more sensitive and, in older women, also more specific to identify pre-malignant cervical lesions (CIN 2, 3; cervical intraepithelial neuplasia) (5–6).

It is therefore of interest to study to what extent high-risk HPV can be identified in normal cervical smears obtained from women prior to a diagnosis of squamous cervical carcinoma.

MATERIALS AND METHODS

At the Department of Pathology and Cytology in Uppsala, all clinical histo-pathological material is collected in a central database (Sympathy, Tietoenator AB, Malmö, Sweden), where each specimen is given an individual topographic and diagnostic code (SNOMED, Systematized NOmenclature of MEDicine). From the database, all cases of squamous carcinoma of the cervix occurring during 2004 to 2005 were collected. Thirty-three cases were identified, of which nine were selected because they all developed cancer in spite of a series of negative cytological smears preceding the tumour diagnosis. All available cytological glass slides from these cases were collected and used for a retrospective analysis of HPV.

In order to perform the polymerase chain reaction (PCR), the specimen (fixated with 70% ethanol) on each glass slide was dissolved in 150 μl DNA extraction buffer (50 mM TRIS-HCl, pH 8.5, 1mM EDTA, 0.5% Tween-20) and 10 μl proteinase K (20 mg/ml). The cells were dislodged from the glass slide and transferred to an Eppendorf tube. This sample was incubated for approximately 2 h at 56°C, after which the proteinase K was inactivated for 5 min at 94°C. Subsequently, 100 μl of saturated ammonium acetate was added. After mixing, followed by 5 min centrifugation at maximum speed, the supernatant was transferred to a new tube and 1.2 ml 99.5% ethanol added. This mixture was incubated at –20°C for at least 30 min and subsequently centrifuged at maximum speed for 30 min. The supernatant was discarded and the pellet was washed with 70% ethanol. After 5 min centrifugation at maximum speed, the pellet was dried and subsequently dissolved in 150 μl water. To detect the HPV L1 gene (the L1 gene encodes the L1 HPV capsid protein), the GP5+/GP6+ primer set was used (product size 150 bp) (7). To ensure that the DNA was properly extracted from the material, a DNA quality test was performed (β-globulin PCR, product size 268 bp) simultaneously. Following the PCR, samples were cleaned with exonuclease I (10 U/μl) and alcaline phosphatase (1 U/μl) by incubation for 15 min at 37°C, 15 min at 80°C, and cooling at 4°C. Subsequently, a sequencing reaction was performed by adding 4 μl BigDye reaction, 9.1 μl Sequence buffer and 0.064 μl 50 μM GP5+ primer to each Eppendorf tube. This mixture was incubated in the PCR machine (25 cycles: 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C). After precipitation of the DNA with NaAc (3 M) and ethanol (95% followed by 70%), the pellet was dissolved in 15 μl Template suppression reagent, prior to the sample being sequenced in the ABI PRISM 3130xl Genetic Analyser (Applied Biosystems) and analysed.

RESULTS

The mean age of the nine women was 48 years, age range 30–75 years (Table I). Together, they had had 68 cytological normal smears, corresponding to 7.5 smears per woman prior to the diagnosis of cervical squamous carcinoma. Fifty-one of the smears were available for HPV analysis and 75% (38/51) were positive for HPV of high-risk type. The nine women all presented with HPV-positive smears between 1.5 and 33 years prior to the diagnosis of cervical carcinoma. The mean value for a retrospective presence of HPV in the women was 13.5 years. Seven women were infected with HPV type 16 and two with HPV type 18. Three of the women with HPV type 16 infections were initially infected with another virus type, 18, 45 or 67.

The mean time lapse between the last smear and tumour diagnosis was 4.4 years, range 0.75–12 years.

Table I. *Human papilloma virus (HPV) analysis of 51 normal cervical smears collected in nine women prior to the diagnosis of invasive cervical carcinoma*

Patients' characteristics	
Age range (years)	30–75
Mean number of normal smears/women	7.5
High-risk HPV positive smears (%)	75
Mean time with an HPV positive reaction (years)	13.5 (range 1.5–33)
Mean time lapse since last smear (years)	4.4 (range 0.75–12)

DISCUSSION

The common feature of the women in this pilot study was that they all had a history of normal smears, mean value 7.5 smears, and that most of their smears (75%) contained high-risk HPV DNA. Some of the women had attended the organized screening only sporadically. Non-participation in screening is known to be the most important risk factor for development of cervical cancer (2, 3). This is evident from the high prevalence of oncogenic HPV among women who choose not to participate in screening (8). Furthermore, as mentioned previously, cytological screening has a low sensitivity, and for that reason a proportion of women develop cervical carcinomas in spite of regular participation in screening (2, 3, 9).

There are several retrospective studies showing that high-risk HPV is present in normal cervical smears before the occurrence of pre-malignant cell alterations. However, these investigations are not based on women with invasive carcinoma, but on cytological material collected from women with a diagnosis of carcinoma *in situ* and treated with cervical cone resection (10–13). In such studies, it is not possible to predict the benefit of primary screening with high-risk HPV tests. In our study, it becomes evident that invasive cervical carcinoma in the women could have been prevented if primary screening with high-risk HPV tests had been performed routinely.

Since HPV tests are more sensitive, they could be used in primary screening in place of cytological screening. An argument against this suggestion is that HPV analysis has a low specificity and a number of women with only transient infections would be subjected to repeated needless examinations. However, this statement is only partly relevant because the prevalence of oncogenic HPV infections decreases with age, and after the age of 50 years it is approximately equal or even lower than the frequency of abnormal cytological smears in the same age category (14). Thus, in middle-aged and older women, primary screening with HPV will be superior to cytology, especially when less expensive HPV tests come onto the market.

The higher sensitivity of HPV tests in comparison with cytological examinations is due mainly to the fact that small amounts of HPV DNA may be present in squamous cells without causing any visible light microscopic alterations. Besides, virons may be present in the cervical secretion outside the exfoliated cells. For this reason, an HPV test may even be positive despite a total lack of human DNA in the smear (unpublished observation).

This short communication is in agreement with several recent investigations and has an important message. It is emphasized that the women in the study would probably not have developed cervical squamous carcinoma at all, or at least the tumour would have been diagnosed at an earlier stage, if HPV tests had been used in the primary screening (6–7). This statement is supported in the present study by the positive test for oncogenic HPV preceding the cervical carcinoma in all the women investigated.

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