

Original Article

HPV-negative Tumors in a Swedish Cohort of Cervical Cancer

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Summary: Despite the common perception that the human papilloma virus (HPV) is a requirement for the development of cervical cancer (CC), a considerable number of CCs test HPV negative. Presently, many countries are shifting to HPV primary CC screening, and it is of importance to increase the knowledge about the group of CCs that test HPV negative. The aim of this study was to reinvestigate a proportion of cervical tumors with a primary negative or invalid test result. Reinvestigation with repeated genotyping (targeting L1) was followed by analysis with an alternative target method (targeting E6/E7) on existing or additional tumor material. Consistently negative tumors were histologically evaluated, and cases with low or lacking tumor cell content, consistent invalid test results, or with suspicion of other than cervical origin were excluded. HPV-negative cases were thereafter subjected to immunohistochemistry (Cytokeratin 5, pan cytokeratin, protein 63, P16, and P53). The HPV-negative proportion could after reinvestigation be reduced by one-half (14%–7%). Additional positive samples were often detected in late polymerase chain reaction cycles, with an alternative (E6/E7) or the same (L1) target, or with a method using shorter amplicon lengths. Confirmed HPV negativity was significantly associated with worse prognosis, high patient age, longer storage time, and adenocarcinoma histology. Some of the HPV-negative cases showed strong/diffuse p16 immunoreactivity, indicating some remaining false-negative cases. False HPV negativity in this cohort was mainly linked to methodological limitations in the analysis of stored CC material. The small proportion of presumably true HPV-negative adenocarcinomas is not a reason for hesitation in revision to CC screening with primary HPV testing. **Key Words:** Uterine cervical neoplasms—Papillomaviridae—Human papillomavirus DNA tests—Formalin-fixed paraffin-embedded tissues—False-negative reactions.

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Cervical cancer (CC) develops as a result of a persistent infection with human papillomavirus virus (HPV). Despite the common perception that HPV is a requirement for the development of CC (1), in many studies, a considerable number of CCs test HPV negative.

The HPV-negative cases have usually been explained by suboptimal study material and methodological limitations. As early as 1999, Walboomers and colleagues showed that, in a worldwide CC cohort with both histologies (mostly SCC, >90%), a rate of 7% HPV-negative samples could be reduced to 0.3%. This was accomplished after analyses with additional detection methods and the exclusion of histologically inadequate samples (1).

In a large international cohort of invasive CC, 15% of the tumors tested HPV negative with a general detection

method. The HPV-negativity rate differs between histologic types, wherein adenocarcinomas (ACs) are generally less likely to test HPV positive compared with squamous cell carcinomas (SCCs) (2). Adenosquamous carcinomas (ASCs) and ACs of the usual type predominantly test HPV positive in contrast to other more unusual morphologic subtypes, such as clear cell carcinomas, serous carcinomas, and endometrioid carcinomas, that are more likely to be HPV negative (3).

Despite the fact that HPV-negative test results often can be explained by technical deficiencies, HPV-negative CCs display distinct characteristics and have been associated with worse patient prognosis compared with HPV-positive CCs (4,5).

In many countries, including Sweden, organized prevention of CC is available with vaccination against HPV in girls together with a national CC screening program. Several studies (6) have shown better protection against CC and its precursors using primary screening with HPV testing compared with cytology screening alone. There is a strong consensus with regard to the implementation of HPV-based screening, and it is already a reality in several regions in Sweden and internationally.

The revision of a prior screening program targeting HPV will be a significant improvement in CC prevention. However, it introduces a risk of missing potential HPV-negative CC. With this in mind, an increased knowledge of the true HPV-negative fraction, also in relation to different histologic types, is needed. A thorough review of the causes of false-negative test results is also warranted in order to further improve the prevention of CC. Furthermore, the incidence of AC is increasing, both in total and in proportion to SCC (7,8), and it is important to examine whether this is true also for the fraction of HPV-negative CC.

Recently published data from a study series of 209 CC cases showed a final result of 7% HPV-negative tumors, this after genotyping with 3 different methods and reassessment of tumor material by a pathologist (9). Here, results from this reinvestigation process are presented together with additional HPV-negative tumor characteristics.

MATERIALS AND METHODS

Of the 209 tumors initially analyzed, 37 samples tested HPV negative or invalid and were included in the reinvestigation group. All women were diagnosed with CC and treated with radiotherapy (external beam radiation and brachytherapy) between 1992 and 2014 at the Department of Oncology at Örebro University Hospital in Sweden. Samples were collected from Örebro

University Hospital, Uppsala University Hospital, and the central hospitals in Eskilstuna, Falun, Gävle, and Karlstad. Staging of the tumors was carried out using the staging system of the International Federation of Gynecology and Obstetrics (FIGO, Montreal 1994), and clinical patient data were obtained from records at the Department of Oncology, Örebro University Hospital. The patient tumor samples were biopsies or surgical excisions retrieved at the time of diagnosis and archived in the form of formalin-fixed paraffin-embedded blocks. Detailed description of the DNA extraction and real-time polymerase chain reaction (PCR) methods used have recently been published (9).

Reinvestigation Process

In the reinvestigation process (Fig. 1), CC tumors with a HPV-negative or invalid result from previously performed genotyping with Anyplex II HPV28 (Seegene) were included ($n=37$). Anyplex is a real-time PCR method targeting 28 genotypes (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 69, 70, 73, and 82) using the viral L1 gene together with the human gene *HBB*. The amplicon lengths are between 100 and 200 bp. A melting curve analysis after 30, 40, and 50 cycles gives a semiquantitative indication of the positive results, but an insufficient (later than 40 cycles) amplification of the human gene results in an invalid test result.

In step 1, all 37 samples were retested with the same method using DNA from the same extraction. Negative or invalid results from the retesting lead to inclusion in step 2. In this step, a second approach was used: an in-house real-time PCR protocol targeting the viral oncogenes E6 or E7 for 12 high-risk and 2 low-risk genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 6, and 11) together with the human gene *ACTB*. Amplicon lengths were between 84 and 134 bp, and PCR curves were manually assessed using a threshold at 35 cycles for a positive result.

Patient tumor samples with consistently HPV-negative results with both real-time PCR methods and with alternative evaluable tumor tissue blocks were analyzed with the same algorithm, as above (step 3, L1x2 followed by E6/E7). Patient samples without alternative tissue blocks or with negative results in the analysis of alternative tumor tissue material were evaluated by a pathologist (step 4). Repeatedly invalid test results led to exclusion of the case after E6/E7 genotyping in step 2.

Finally, in step 4, the remaining HPV-negative samples were investigated by a pathologist. After the final assessment, samples with lacking tumor amount and quality or with a suspicion of other than cervical origin were excluded.

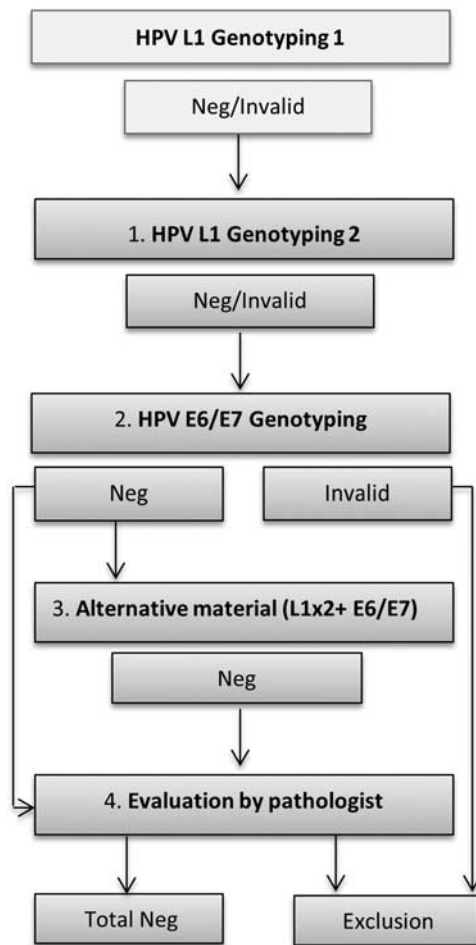


FIG. 1. Flowchart of the reinvestigation chain. Samples previously tested negative or invalid (Anyplex™ II HPV28, targeting L1) were reanalyzed with the same method in step 1 of the reinvestigation chain. In step 2, an alternative target method (in-house real-time PCR, targeting E6/E7) was used. Persistently human papilloma virus (HPV)-negative samples with alternative tumor tissue block were in step 3 analyzed with the same algorithm (L1*2+E6/E7) as above. The remaining HPV-negative cases were evaluated by a pathologist in step 4. Consistent invalid test result, lack of tumor in sample, and suspicion of other than cervical origin led to exclusion.

Immunohistochemistry and Histologic Evaluation

All cases were histologically reviewed on eosin-stained slides and, when appropriate, reclassified according to the most recent WHO classification (10) including the subclassification of ACs. The HPV-negative cases were thereafter subjected to immunohistochemistry [Cytokeratin 5 (CK5), pan cytokeratin (panCK), protein 63 (P63), P16, and P53] in order to (A) confirm squamous differentiation by determining CK5 and p63, or (B) describe the immunoreactive pattern of p16 and p53.

In brief, immunohistochemistry staining was performed (Table 1) on 4 µm formalin-fixed paraffin-embedded tissue sections on automated staining systems, Omnis (Agilent Technologies, CA) or Benchmark ULTRA (Ventana Medical Systems, AZ) from the respective manufacturer. Staining was carried out according to the manufacturer's instructions.

Exclusion Criteria

Tumors were excluded after histologic evaluation according to 3 reasons: (A) inadequate tumor material (quality and/or remaining percentage) in the block on morphologic examination performed on a slide cut after material for molecular analysis had been used, (B) review of clinical data showing a preexisting noncervical malignancy likely to be the origin of the tumor or previously performed (> 10 yr) complete hysterectomy, or (C) consistent invalid test results.

Detection of HPV With General L1, MGP5+/6+ Primers

In addition, the samples (n = 30, except the 7 excluded samples) were finally analyzed using the L1, MGP5+/6+ primer system (11). This was performed to further investigate possible reasons for conflicting results in the reinvestigation process and to examine the negative samples using a general method. Analyses were carried out in sample duplicates of 25 µL together with negative

TABLE 1. Immunohistochemical method overview

Antibody	Clone	Manufacturer	Ready to use (RTU)/dilution	System	Evaluation criteria
P16	E6H4	Ventana	RTU	Ventana	Positive (intense nuclear/cytoplasmatic) > 25%; indeterminate and negative
CK5	XM26	Leica	1:100	Omnis	Tumor cells positive
panCK	AE1/AE3	Agilent	RTU	Omnis	Tumor cells positive
P63	DAK-P63	Agilent	RTU	Omnis	Tumor cells positive
P53	DO-7	Agilent	RTU	Omnis	Tumor cells positive, > 30%

Immunohistochemical analysis was performed on all HPV-negative cases (n = 14) with the purpose to confirm squamous differentiation and/or describe the immunoreactive pattern of p16 and p53 in this group. Staining was performed on an automated staining system from the respective manufacturer.

CK5 indicates Cytokeratin 5; P63, protein 63; panCK, pan cytokeratin.

and positive controls for both MGP and the human control gene *HBB*. Sample reactions contained 1x Quantitect SYBR green master mix (Qiagen, Germany), either 0.3 μM forward and reverse primers for *HBB* or 0.3 μM forward and reverse MGP primer mix and DNA of ~50 ng, and were manually applied on 96-well plates. Subsequent analysis was performed on the 7500 fast real-time PCR system (Applied Biosystems, the Netherlands). The standard program was used with a denaturation step at +95°C for 10 min, followed by 5 cycles at +95°C for 0.5 min, +42°C for 0.5 min, and +72°C for 0.75 min; 45 cycles at +95°C for 0.5 min, +64°C for 0.5 min, and +72°C for 0.75 min; and a final step at +72°C for 10 min. The software 7500 fast system SDS (Applied Biosystems) was used to analyze results, and the curves were manually assessed using a threshold at 35 cycles for a positive result. Every run was analyzed with a positive and a negative control.

Ethical Approval

The study was approved by the regional ethical committee board in Uppsala, Sweden (D nr 2008/122).

Statistical Analyses

Kaplan-Meier method was used for survival analyses, in which comparisons were made using log-rank analysis. Comparisons of proportions were performed with Pearson

χ^2 test, and means were compared with the independent *t* test. Multivariate analyses of prognostic factors were made with Cox proportional hazards regression method. For all statistical tests, *P*<0.05 was considered statistically significant. The Statistica software (version 13, 2015; StatSoft Inc., Tulsa, OK) was used for the statistical analyses. Percentages have been rounded to the nearest integer, which may result in totals other than 100%.

RESULTS

Patient and Tumor Characteristics

All 37 patient tumor samples previously tested negative or invalid from a cohort of 209 women diagnosed with CC and treated with radiotherapy were reinvestigated. In the present study group, the mean age was 66 yr (range: 31–90). The histologic distribution of the tumors was as follows: SCC (51%, 19/37), AC (43%, 16/37), and ASC (5%, 2/37), and most tumors were FIGO stage II (51%, 19/37), followed by stage I (27%, 10/37), stage III (11%, 4/37), and stage IV (11% 4/37).

Reinvestigation Resulted in a Drop in HPV Negativity From 14% to 7%

Previous results from HPV genotyping using Anyplex HPV28 showed a proportion of 14% (30/209)

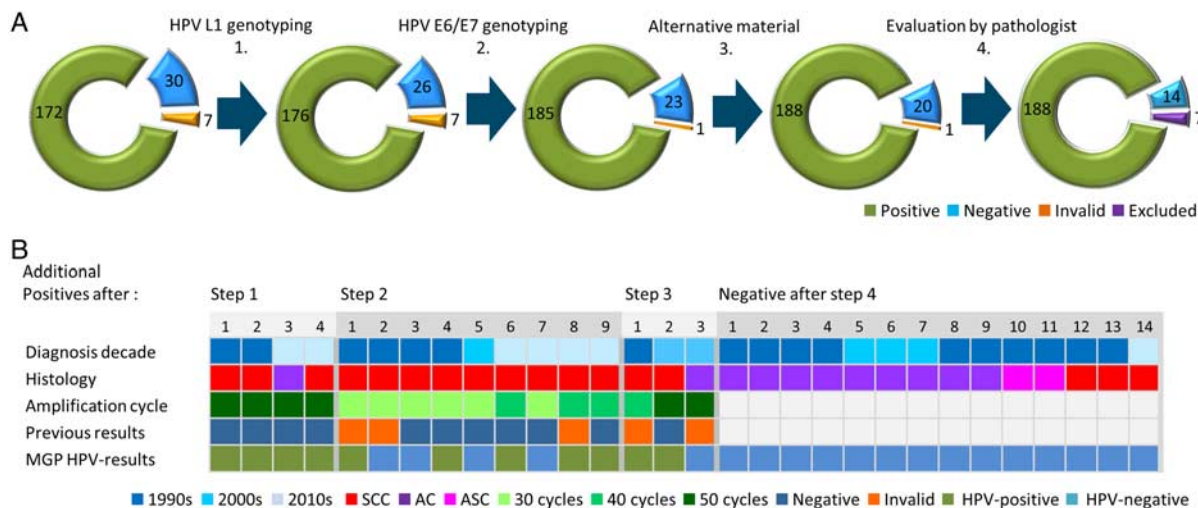


FIG. 2. The reinvestigation process of 37 tumors previously tested human papilloma virus (HPV) negative or invalid. (A) All samples were retested with the same method (step 1), and consistently negative samples were tested with an alternative target method (step 2). Cases with alternative tumor tissue available were analyzed with both methods, L1*2+E6/E7 (step 3) and repeated negative results led to evaluation by a pathologist (step 4). Reasons for exclusion were consistent invalid test result, lack of tumor material, or suspicion of other than cervical origin. (B) Sixteen additional samples tested positive in steps 1 to 3 of the reinvestigation chain, and, after step 4, 14 samples still had a HPV-negative test result. An overview of sample storage time (diagnosis decade), histology of the tumor, amplification cycle for positive result in reinvestigation, result previous to reinvestigation, and results from additional HPV analysis using MGP is shown in the color chart.

HPV negativity; seven samples were invalid in the first run. With repetitive analysis in this study (step 1), the second approach with alternative genotyping method (step 2), and alternative material (step 3), the HPV negativity dropped to 10% (20/209) (Fig. 2A). After assessment of tumor material together with immunohistochemistry, 6 samples were excluded. Of these, 4 samples lacked tumor cells, 2 cases were excluded due to complex clinical history wherein a noncervical origin could be suspected, even though the clinical presentation was with a cervical/paracervical tumor mass. Together with the one excluded case due to consistently invalid HPV, the final total number of excluded samples was 7. This gave an HPV-negative proportion of 7% (14/202).

Additionally Detected HPV-positive Samples were Typically SCCs, Found in Late PCR Cycles or With Alternative Genotyping Method

Through steps 1 to 3 in the reinvestigation chain, an additional 16 samples tested HPV positive (Fig. 2B). Of these samples, 9 were added after targeting an alternative viral gene, 4 samples by rerunning with the same method, and 3 samples by running the same method on DNA from an alternative tissue block. The additional positive samples were typically SCC (12/14). When detected by repeating the same method, positive results were observed in late PCR cycles; however, when detected with an alternative viral target, this did not apply. The genotype distribution in additionally detected positive samples was as follows: HPV16 (n=2), HPV18 (n=3), HPV33 (n=1), HPV39 (n=1), HPV45 (n=7), HPV51 (n=1), and HPV18+33 (n=1).

At the end of the reinvestigation, 14 cervical tumor samples persisted with an HPV-negative test result. These were most often samples that had been archived since the 1990s (10/14, 71%), and the group predominantly consisted of AC, 64% (9/14 ACs, 2/14 ASCs, and 3/14 SCCs).

Further Analysis Using a General L1, MGP5+/6+ System, did not Result in Additional Positive Samples

All cases in the reinvestigated group, except the 7 formerly excluded samples, were analyzed using the MGP5+/6+-primer system. This did not result in any additional HPV positivity. However, 11 of the 16 additional positive samples detected in the reinvestigation previously conducted were also positive using this method (Fig. 2B).

Histologic Evaluation Led to a Slight Change in Histologic Distribution in the Total Cohort

After the histologic evaluation and reclassification, the histologic distribution in the total cohort (n=202, not counting the 7 excluded cases) slightly changed. The distribution was as follows: SCC, n=169 (84%); AC, n=27 (13%); ASC, n=4 (2%); and small cell neuroendocrine carcinoma (SCNC), n=2 (1%). The AC subtypes presented were as follows: usual AC, n=9; not otherwise specified (NOS), n=4; mucinous NOS, n=2; gastric, n=4; villous, n=3; clear cell, n=2; and serous, n=3.

HPV-negative SCC (n=3) and ASC (n=2) were confirmed concerning epithelial and squamous origin with immunohistochemistry using panCK marker, CK5, and p63 standings, respectively. Four of 5 tumors showed squamous differentiation, while 1 tumor only showed a panCK marker; morphologically, this case was classified as a glassy cell carcinoma (12), a subgroup of ASC according to the present WHO classification.

Concerning HPV-negative AC (n=9), 4 were of usual morphologic type, 2 serous, and 1 case each of NOS, gastric type, and villoglandular morphology, respectively (Table 2).

Combining genotyping data for this cohort published elsewhere (9) and the new histologic classification, ACs were HPV positive in 67% (18/27). Among the usual subtype cases, 56% (5/9) tested positive, and, in the group with unusual histologic subtypes corresponding to the subtypes represented in the HPV-negative proportion (AC NOS, gastric, villous, and serous)

TABLE 2. *p16 and p53 immunohistochemical analysis was performed on all confirmed HPV-negative samples (n = 14)*

No			P16	P53
1	AC	NOS	+	-
2	AC	Usual type	+	-
3	AC	Usual type	(+)	-
4	AC	Usual type	(+)	-
5	AC	Usual type	-	-
6	AC	Gastric type	-	+
7	AC	Villoglandular	(+)	+
8	AC	Serous	+	+
9	AC	Serous	+	-
10	ASC	Glassy cell	+	
11	ASC	Glassy cell	+	
12	SCC		+	
13	SCC		+	
14	SCC		+	

The HPV-negative cases were of varying histologic categories, specified in the table.

AC indicates adenocarcinoma; ASC, adenosquamous carcinoma; NOS, not otherwise specified; SCC, squamous cell carcinoma; +, strongly positive staining; (+), intermediate staining; -, negative.

(9/13), 69% tested positive. Among all nonusual ACs (AC NOS, mucinous NOS, gastric, villous, clear cell, and serous) (13/17), 72% were HPV positive.

Immunohistochemical P16 Expression in Samples Tested HPV Negative Indicates a Remaining Fraction of False-Negative CC

Among the HPV-negative SCCs and ASCs, all 5 cases were considered p16 positive. In the AC group (n=9), p16 staining was positive in 4, indeterminate in 3, and negative in 2 cases, respectively, with no correlation to histologic subtype (Table 2). Immune reactivity for p53 was considered to be positive in 3 of 9 and negative in 6 of 9 of the HPV-negative AC.

HPV Negativity was Associated With a Higher Mean Age, AC Histology, and Longer Sample Storage Time

In the whole cohort (n=202), women with tumors that tested HPV negative had a significantly higher (*t* test; *P*=0.016) mean age (70.3 yr) compared with women with tumors that tested HPV positive (59.5 yr) (Table 3). Tumor size and stage distribution did not differ between these 2 groups; however, the type of histology (Pearson χ^2 test; *P*<0.00001) and decade of diagnosis (Pearson χ^2 test; *P*=0.001) did significantly differ between the HPV-positive and HPV-negative groups. Samples stored since the 1990s were more common (71%) in the HPV-negative group compared with the HPV positive (26%).

Patients With an HPV-negative Tumor had a Worse Prognosis, Which was Associated With the AC Histology Rather than With HPV Negativity

The primary cure rate of the complete series was 95%. No significant association between primary cure rate and HPV status was found (HPV positive vs. HPV negative; χ^2 , *P*=0.095). The recurrence rate of the complete series was 29%, with mainly distant recurrences (20%). There was a numeric difference in recurrence rate between HPV-negative tumors (50%) and HPV-positive tumors (27%); however, it did not reach a significant difference (χ^2 test; *P*=0.061) (Table 3). Cancer-specific survival rate at 5 yr was significantly (log-rank test; *P*=0.009) worse for patients with HPV-negative tumors (27%) compared with HPV-positive tumors (69%). However, when comparing only patients with AC histology (HPV positive vs. negative), this does not apply (log-rank test; *P*=0.667).

In a Cox proportional hazard regression analysis, AC histology was an independent prognostic factor

TABLE 3. Patient and tumor characteristics in the whole study cohort: HPV-positive versus HPV-negative tumors

	HPV positive	HPV negative	Statistics
Mean age (yr)	59.5	70.3	<i>t</i> test; <i>P</i> =0.016
Mean tumor size (mm)	42.0	45.2	<i>t</i> test; <i>P</i> =0.383
Type of histology			
SCC	166	3	χ^2 test; <i>P</i> <0.00001
AC	18	9	
ASC	2	2	
SCNC	2	0	
Total	188	14	
Tumor stage			
Early stage (FIGO I–II)	157	12	χ^2 test; <i>P</i> =0.830
Advanced stage (FIGO III–IV)	31	2	
Total	188	14	
Primary cure rate	96%	86%	χ^2 test; <i>P</i> =0.095
Overall recurrences	27%	50%	χ^2 test; <i>P</i> =0.061
Cancer-specific survival rate (5 yr)	69%	27%	Log-rank test; <i>P</i> =0.009
Diagnosis decade			
1990s	49	10	χ^2 test; <i>P</i> =0.001
2000s	77	3	
2010s	62	1	
Total	188	14	

The HPV-positive (n=188) and HPV-negative (n=14) groups showed significant differences in mean age, type of histology, age of tumor material analyzed, and cancer-specific survival rate at 5 yr. However, tumor size, stage, primary cure rate, and overall survival did not significantly differ between the groups.

AC indicates adenocarcinoma; ASC, adenosquamous carcinoma; NOS, not otherwise specified; SCC, squamous cell carcinoma.

[hazard ratio=4.232 (95% confidence interval: 2.184–8.200)] (AC vs. SCC), while HPV status (HPV positive vs. HPV negative) was not [hazard ratio=1.004 (95% confidence interval: 0.421–2.393)].

DISCUSSION

The results in the present study are consistent with data from previous studies, wherein extended analyses of HPV-negative CC with an alternative method and viral target give reduced HPV-negative proportions (5,13). There are methodological aspects to consider in relation to HPV-negative CC. The HPV-negativity rate has been reported to vary between geographical regions, histologic subtypes, patient age, and material storage time (14). A negative test result can be attributed to a variety of reasons, which of several could give false-negative results. One of them is tumor sample quality, which is of importance, especially when working with formalin-fixed paraffin-embedded material wherein protein cross binding and nucleic acid degradation over time (15) could affect the

analysis. We saw a significant difference ($P=0.001$) in storage time between tumors that tested HPV positive ($n=188$) and those that tested HPV negative ($n=14$) after reinvestigation, and the majority of the HPV-negative cases (10/14) was stored since the 1990s. This is in line with previous reports, wherein longer storage time was associated with an HPV-negative test result (14).

It has previously been described that loss of the frequently targeted viral L1 gene after viral integration could be a reason for HPV-negative test results (16). In the present study, 9 of 16 additional positive samples were detected by targeting the viral oncogenes E6/E7. By running these samples with a third method (MGP5+/6+), again targeting L1, this cause could be dismissed in 5 of 9 cases that tested HPV positive with a second L1 method. As L1 was not detected in 4 samples, loss of L1 is a possible explanation for the initial negative test result in these tumors. A difference in the results between the L1 methods might also relate to genomic variances in primer/probe regions.

HPV detected by repeating Anyplex (L1) genotyping (in the same or alternative tissue block), in steps 1 and 3, was most often observed in late PCR cycles. This could be a sign of degraded DNA, but also of low viral load. However, in this case, it seems to be a methodological matter, as all but one case tested positive using MGP5+/6+ (L1) primers in earlier PCR cycles. The overall somewhat shorter amplicon of MGP compared with Anyplex (150 bp vs. 100–200 bp) could be a decisive factor.

The genotype distribution in the reinvestigated group differed in some genotypes from the total cohort (previously published data). Especially, HPV45 was significantly more common in the reinvestigation group (7/16, 44%) compared with the whole cohort (17/218, 8%) and was often detected in late PCR cycles. It is unlikely that this finding of additional HPV45 positive cases would be attributed to contamination during analysis, as most of the cases also were analyzed, and HPV45 positive, in routine practice at the time of diagnosis (data not shown). HPV16 was in contrary to HPV45 more commonly detected in the whole cohort (93/218, 43%) compared with the reinvestigation group (2/17, 12%). These findings indicate sensitivity variations in the used methods for certain genotypes.

Low tumor amount in the sample has previously been described as the explanation for a significant proportion of HPV CCs testing negative and extracervical tumor origin as a minor part (1). This was also an issue in this cohort, and 7 samples were excluded

from the initially 37 cases in the reinvestigation chain: 4 due to low tumor amount and 2 due to suspicion of other than cervical origin.

Cervical AC differs from SCC in several aspects. Cervical AC more often contains HPV 18 and 45 (2) and have been associated with a higher rate of lymph node spread, ovarian involvement, and distant metastasis (17–19). Cervical AC does also seem to be less radiosensitive compared with SCC (20). In most studies, regardless of tumor material and detection methods, a proportion of CCs test HPV negative. The HPV-negative cases are predominantly ACs; however, there are a few reports of carefully investigated HPV-negative SCC cases, often well differentiated, keratinizing, and not believed to be preceded by CIN (21,22). After reinvestigation in the present study, 14 HPV-negative tumors remained. Most were of AC histology (4 usual, 1 NOS, 1 gastric, 1 villoglandular, and 2 serous), 2 of glassy-type ASC, and 3 of SCC. In large epidemiological studies, HPVs are commonly detected in usual-type AC (72–90%), while HPVs are more infrequently found in the unusual morphologic subtypes, ranging from 0% to 30% (3,14). In the present study, an HPV detection rate of 67% AC was presented, wherein 56% of the usual ACs are HPV positive, and 69% of the nonusual subtypes were HPV positive. The HPV-positivity rate in this cohort differs from larger studies; here, usual ACs harbor fewer HPVs in comparison with the unusual group. However, the cohort is small, with very few numbers of cases per histologic subtype, which makes proportional comparisons difficult.

Data presented by Molijn et al. (23) suggest that the HPV-positivity rate presented in ACs in previous studies could be overestimated. They compared HPV presence in DNA extracted from CC tumor samples using whole-tissue sections and laser capture microdissection. Analysis from laser capture microdissection showed lower HPV positivity, and the HPV could in some selected example cases be located in adjacent cervical mucosa. In the unusual AC (AC NOS, minimal deviation/gastric, clear cell, endometrioid, and serous), only 0% to 24% of the samples formerly positive with whole-tissue sections remained HPV positive with laser capture microdissection. This could mean that unusual ACs largely develop HPV independently.

To further evaluate the HPV-negative CC group in the present study, immunohistochemistry for p16 and p53 was performed. P53 protein levels are usually decreased in HPV-related cancers due to HPV E6 induction of p53 deregulation. Thus, p53 is usually

not mutated in CC (29). Immunostaining of p53 has been shown to be greater in HPV-negative cervical AC than in HPV-positive AC cases (30). P16 is a known and often used surrogate marker for HPV, and, in this cohort, all SCCs and ASCs with a HPV-negative test result had a strong/diffuse p16 expression. A few previously published studies with regard to keratinizing, well-differentiated carcinomas, confirmed the presence of HPV-negative SCC, wherein the cases were either not p16 analyzed or were completely p16 negative (22). None of the HPV-negative SCC tumors in this cohort showed characteristics similar to that described, and one case had previously in routine practice tested HPV16 positive (data not shown). Thus, p16 positivity in the SCC and ADC cases indicated that they were HPV associated and most likely had a false-negative HPV test result.

In the HPV-negative cervical AC presented here, both usual and unusual AC cases were represented. Four samples were strongly/diffusely p16 positive (2 serous and one each of usual and NOS). The strong p16 reactivity observed in the usual and NOS AC could very well be a sign of HPV-induced CC (24,25). In the 2 serous cases, a p16 positivity is not necessarily a sign of HPV association. On the contrary, it has been reported that serous ACs are most often HPV negative, p16 and p53 positive, with some exceptions (26). The remaining 5 tumors showed intermediate or totally absent p16 staining (3 usual and one each of villous and gastric subtypes). Gastric-type ACs have been reported to be mostly HPV negative, and typically p16 negative and p53 positive (10), and this was true for the one case in this group. The 4 samples of usual and villous subtypes, belong to histologic categories that most often are known to be HPV positive, p16 positive, and harbor wild-type p53 with intermediate or low p53 staining (27). A negative p16 or a positive p53 immune result in these samples could indicate an HPV-independent CC, but it is highly speculative.

HPV negativity in CC has been linked to poor prognosis (5,28–30), as in the present study. However, the significant association was lost in a multivariate analysis wherein AC histology seemed to be the crucial factor influencing survival. When comparing within AC alone, there was no significant difference in cancer-specific survival rate between HPV-positive and HPV-negative cases. Previous results are conflicting on whether HPV negativity alone is a determining factor for patient prognosis. In other HPV-related malignancies, such as head and neck (31) and vaginal cancer (32) treated with radiation, HPV positivity was

also shown to be associated with a favorable prognosis. This has been attributed to molecular differences between the groups and that HPV-positive tumors are believed to be more radiation sensitive.

An additional possible explanation for HPV-negative CC is presented by Banister et al. (33) wherein HPV-initiated cancer could become HPV independent in later stages. The presented data show a subset of HPV-positive CC with low or missing E6/E7 oncogene expression that they termed HPV-inactive CC. This group presented gene expression and viral and somatic methylation pattern that differed from HPV-active tumors. The contrast between the 2 groups did in several aspects mirror the differences between HPV-positive and HPV-negative CC. Their findings may describe a fraction of initial HPV-dependent tumors that could develop to be HPV negative. Further research in this field could focus on the investigation of archival screening material from the patients with HPV-negative CC in order to better understand the HPV relation to the cancer development in these cases.

In addition detected HPV positives in this cohort (HPV 16, 18, 33, 39, 45, 51) were all of IARC 1 classified genotypes. This is reassuring in that they are covered by all commonly used HPV-screening methods. However, as these are established cancer cases and the false HPV negativity is most likely a result of long-time storage and methodological limitations, it is not representative of a screening situation.

The prevalence of true HPV-negative CC precursors has recently been investigated. Petry et al. (34) undertook an evaluation of HPV-negative CIN2+ (CIN2, CIN3, AIS) in the ATHENA study, wherein all negative results could be explained as false-negative CIN2+ (HPV positive with another method, CIN2+ false positive, or immune reactivity for p16), and they could not find any signs of true HPV-negative CIN2+. This supports the idea that HPV-negative CCs in some cases are not preceded by precursors, alternatively with a rapid progress with precursors that is difficult to detect and could potentially be missed in screening.

With a considerable number of ACs and rare cases of SCC, confirmed negative in CC studies, the question of detection degree of a screening program with primary HPV is raised. With this in mind, screening with primary cytology has lower detection in AC compared with SCC (35) and screening with primary HPV is expected to improve the prevention of AC in CCs (6). Thus, neither HPV-negative precursors nor HPV-negative ACs are a reason for

hesitation when it comes to changes from primary cytology to primary HPV screening in CC prevention. However, more knowledge of this small group of HPV-negative carcinomas is of value to future additional improvement of the prevention of CC.

CONCLUSIONS

The proportion of HPV-negative tumors in this cohort could be reduced by one-half (14%–7%), after reinvestigation of HPV-negative and invalid samples. Confirmed HPV negativity was in this group significantly associated with worse prognosis, high patient age, longer storage time of the tumor tissue, and AC histology. The prognosis difference between HPV-positive and negative CC seems to depend on histology rather than HPV status. Part of the HPV-negative cases show strong/diffuse p16 immunoreactivity indicating some remaining false-negative cases.

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