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Genotyping of Human Papillomavirus in triaging of low-grade cervical cytology

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Article condensation

Typing for HPV16 or for HPV16/31/33 can improve the predictive ability of HPV-based triaging of low-grade cervical cytology.

Short version of article title: HPV typing in triaging of low-grade cytology.

Abstract

Genotyping of Human Papillomavirus in triaging of low-grade cervical cytology

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Objective: To evaluate whether typing of human papillomavirus (HPV) among women with low-grade cervical cytology can improve the ability to identify women with cervical cancer or Cervical Intraepithelial Neoplasia III (CIN III+).

Study design: 1595 women with low-grade cervical cytology participating in a randomised implementation trial of HPV triaging using Hybrid Capture II were also HPV-genotyped and CIN III+ predictive values evaluated.

Results: HPV 16 was detected in 57% of CIN III+ cases, but only among 24% of all tested women. Testing for the 3 HPV types with highest risk (HPV16/31/33) detected 77% of CIN III+, with 36% of women testing positive. Positivity for the other "high-risk" HPV types had a decreased risk for CIN III+.

Conclusion: Different "high-risk" HPV types confer different risks for presence of CIN III+, implying that HPV-genotyping could be useful for optimization of triaging strategies.

Key words: HPV-genotyping, low-grade cervical cytology, triaging.

Introduction

Women with cervical intraepithelial lesions that confer an increased risk of cervical cancer are identified by cytological screening[1]. Policies for follow-up after finding atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesion (LSIL) in cytology vary from repeat cytology[2-4] to immediate referral for colposcopy and biopsy[5-8]. Referral of all women with ASCUS/LSIL in cytology to the colposcopy clinic and the subsequent histological examination yields substantial costs for the health care system[9] and often creates feelings of anxiety and discomfort for the women concerned[10, 11]. Given the key etiological role of oncogenic human papillomavirus (HPV) infections in the development of cervical cancer[12], HPV testing is useful as a triage method to select women at increased risk of cervical cancer, thus justifying referral for colposcopic exploration[13, 14]. A major 3-way randomised trial comparing HPV triaging, repeat cytology and colposcopy of all women found that repeat cytology was inferior for managing ASCUS smears and that HPV triaging and colposcopy of all women were equivalent in terms of safety[15].

The Hybrid Capture II method (HCII) is one of only 2 HPV testing methods approved by the United States Food and Drug Administration (US FDA). The HCII contains cocktails of type-specific probes, one “low-risk” (LR) mix and one “high-risk” (HR) mix containing 13 different HPV so-called “high-risk” HPV types. HCII does not provide information regarding the specific HPV type. Since different so-called “high-risk” types have substantially different risks for CIN III and cancer[16-26], HPV genotyping should be relevant for HPV triaging. We have compared the key test performance indices of an HPV test without typing (HCII) to test results for specific HPV types obtained using general primer PCR with GP 5+/6+ primers followed by bead-based multiplex-genotyping on the Luminex platform.

Materials and methods

Study design

In Sweden, all women aged 23-49 years are invited for cervical cancer screening at 3-yearly intervals and women aged 50-60 at 5-yearly intervals. All women resident in the Stockholm County, Sweden, who on their invitational smear had the cytological diagnoses ASCUS or LSIL between 17th March 2003 and 16th January 2006 were included in a randomised health care policy as previously described [27], which was approved by the Ethical Review Board in Stockholm. Sweden uses the old North American cytological terminology where CIN grade I (CINI) is also used as a cytological diagnosis. This corresponds closely to the LSIL in modern terminology and CIN1 in cytology has therefore been substituted for LSIL throughout this paper. The policies compared were 1) referral of all women with ASCUS or LSIL for colposcopy and biopsy (previous policy) and 2) HPV-based triaging referring all women with ASCUS or LSIL for a new visit with HPV-testing using HCII. All the 15 ObGyn clinics in Stockholm County were randomised to either colposcopy of all women (1567 women with ASCUS/LSIL) or to HPV triaging (1752 women with ASCUS/LSIL). The present study focuses on the women randomised to the HPV-triaging arm, where 1600 samples could be analyzed with HCII. We extended the HPV testing to also include HPV typing of these samples. As 5 samples were missing, we obtained HPV typing data on 1595 samples. The mean age of the women was 33 years (min 23, max 61). The HCII-positive women (N=1154) were referred for a colposcopy-directed biopsy, and the HCII-negative women (N=441) were referred for repeat cytology 12 months later. In the HPV triaging arm, a total of 1917 colposcopies were performed and 2766 biopsies were obtained. All histopathologies were interpreted on a routine practise basis.

HPV DNA testing

At enrolment, all samples were tested by HCII (Qiagen, Hilden, Germany), a pool-probe, signal amplification DNA test that targets a group of 13 high risk HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). HCII does not provide information on the specific genotypes present. The samples were taken using the HCII DNA specimen collection

kit (according to the manufacturer´s instructions) and stored frozen in Specimen Transport Medium (STM). HCII was performed according to the manufacturer´s instructions.

DNA extraction

DNA from the samples stored in STM-buffer were extracted using SDS/proteinase K. 150 µl lysis buffer (10mM Tris-HCl, 10 mM NaCl, 10 mM EDTA, 4% SDS at pH 7.8) with addition of 4% proteinase K was added to 50 µl of each sample and incubated at 37 °C overnight. After addition of 75 µl saturated NH_4Ac the samples were centrifuged for 15 min at 16000 x g, the supernatant was transferred to a new tube and the DNA was precipitated with 450 µl of absolute ethanol at -20 °C for 30 minutes followed by 5 min centrifugation at 16000 x g. The DNA pellet was washed once with 250 µl 70 % ethanol, the dry pellet was dissolved in TE-buffer (10mM Tris-HCl, 0,1mM EDTA pH8.0) and stored at -20 °C until analysis.

HPV DNA genotyping

PCR using the general primer pair GP5+/bioGP6+, was performed as previously described [28]. Briefly, 1 µl of extracted DNA was added to the PCR master mix in a final volume of 25 µl. The PCR was performed in an Eppendorf Mastercycler epgradient (Eppendorf, Denmark), the first step at 94°C for 10 min was followed by 45 cycles of denaturing at 94°C for 1.5 min, annealing at 50°C for 2.0 sec and 40°C for 1.5 min with a 7% ramp between 50°C and 40°C of 0.2°C/second, an extension step at 72 °C for 2 min, and a terminal extension step at 72 °C for 4 min. Positive controls were 10-fold dilutions, 1-0.01 ng/µl, of HPV 16 DNA purified from SiHa cells in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, 10 ng/µl human placenta DNA, pH 8.0).

HPV detection and genotyping was performed using multiplex bead-based hybridization with Luminex technology as described by Schmitt et al [29]. The probes for the HPV types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 45, 51, 52, 56, 58, 59, 66, 68, 70, 73, 82, and an HPV 35 variant sequence (5'CTG CTG TGT CTA CTA GTG A 3') were used. The cut-off for positivity was set individually for each included HPV type to two times the mean MFI of 12 negative water controls tested on each 96 well plate.

The samples were tested for amplifiability by real time PCR amplification of the human β-globin gene as previously described [30] using 0,2 µM of modified PCO3 and PCO4 primers (PCO3 14-39F: 5'-ACACAACTGTGTTCACTAGCAACCTC -3', PCO4 123-103R: 5' -

CCAACTTCATCCACGTTCACCT -3') and 0.04 μM Taqman probe (β-globin 55-85: 5' – FAM-TGCACCTGACTCCTGAGGAGAAGTCTGC-TAMRA -3') and 5 μl of sample in a 25 μl reaction.

Statistical analysis

Odds ratios (OR) with 95% confidence intervals (CI) were calculated using binomial logistic regression using the software R v2.7.2 (www.r-project.com).

Results

Altogether, 1154/1595 women were HPV-positive by HCII, whereas the PCR-based genotyping method found 1148/1595 women positive for any HR type and 140/1595 positive for a LR type (not tested for by HCII) (table 1). The sensitivities for CIN II or worse (CIN II+) and CIN III or worse (CIN III+) in histopathology were 97.1% and 97.8% with HCII, respectively, and 95.0% and 96.1% with PCR, respectively (table 1). The few cases of CIN II+ and CIN III+ who were positive in HCII but not in PCR for the 14 HR types all contained LR HPV types (data not shown).

The single HPV type with the highest sensitivity for CIN II+ and CIN III+ was HPV 16, detected in 42.2% of CIN II+ cases and 57.2% of CIN III+ cases, but with only 23.8% of women testing positive. When combining the 3 HPV types with highest probability for CIN III+ (HPV 16/ 31/33) the sensitivity for CIN III+ in histopathology was 76.7%. Inclusion of HPV 18 raised the sensitivity to 79.4% (table 1). The highest positive predictive value (PPV) for CIN III+ for a single HPV-type was observed for HPV 16 (27.1%). The combinations of HPV 16/18 and of HPV 16/31/33 also had high PPV (22.5% and 23.8%, respectively). The PPVs for any HR type and for HCII were considerably lower (15.1% and 15.2%, respectively).

The HPV type with highest odds ratio (OR) for CIN III+ was HPV 16 (OR 5.55 95% CI 4.01-7.68). Both HPV 31 (OR 2.10 95% CI 1.37-3.22) and HPV 33 1.91 (OR 1.91 (1.10-3.32) significantly increased the probability that a test-positive woman would have CIN III+ (table 2). For a joint HPV 16/31/33 test, the risk for CIN III+ was quite high (OR 7.38 95% CI 5.11-10.64). Addition of HPV 18 did not further increase the risk (OR 6.63 95% CI 4.53- 9.70) (table 2). The HR HPV types other than 16, 18, 31, and 33 were only found in 25.2% of CIN II+ cases and 16.7% of CIN III+ cases (table 1). When using CIN III+ as outcome, no other HPV type except HPV16, 31 and 33 was associated with significantly increased risk (table 2), with most of these so-called “high-risk” types actually having point estimates of CIN III+ risk that were below unity (table 2). Adjusting for co-infection with other HPV types, age or cytological diagnosis resulted only in marginal changes of the estimates (table 2).

When comparing CIN II+ and CIN III+ as the outcome, HPV 16 was the only HPV type that had a higher risk for CIN III+ than for CIN II+. For all other HPV types, the point estimate of risk was decreased for CIN III+ compared to CIN II+ (table 2).

A comparison of the ORs for CIN III+ between the HCII test and the PCR test ("any high-risk type") showed that the OR was higher for the HCII test (19.37 (95% CI 7.14-52.54)) than for the PCR test (10.84 (95% CI 5.05-23.28)). Including low-risk HPV types in the definition of positivity in PCR did not increase the OR (10.47 (95% CI 4.27-25.68)) (table 2).

Age had only a limited effect on the risk for CIN II+, and this risk was explained by adjusting for HPV (table 2). The cytological diagnosis (ASCUS or CIN I) at the study baseline did not confer any significant difference in risk for CIN II+ or CIN III+ (table 2).

Comment

We have evaluated the usefulness of HPV typing in triaging of women with ASCUS/LSIL in cytology. HPV 16 positivity was associated with a higher risk for CIN II+ and CIN III+ than any other HPV type, but testing only for HPV 16 had a rather low sensitivity (57.2%) for detecting the women with CIN III+. A combination of testing for HPV16 with testing for the other 2 HPV types that were associated with increased CIN III+ risk (HPV 31 and 33) gave a higher PPV, acceptable sensitivity (76.7%) and required referral of only half as many women (36%) as a strategy based on the HCII test (72%). Reducing the number of women being referred for colposcopy/biopsy is important both for saving health care resources and for reducing unnecessary anxiety, and it is therefore important to refer as few as possible of the women who have only a low risk for CIN III+.

When evaluating the usefulness of a diagnostic test result, it is important to compare the probability of disease before and after the testing. In the cohort genotyped in this paper, the pre-test probability of CIN III+ was 11.2%. For most of the HR HPV types, a positive test result resulted in a decreased (<11.2%) post-test probability of CIN III+ (equivalent to an OR of less than 1). The decreased probability was not statistically significant for any individual HR type, but there was a highly significantly decreased post-test probability for CIN III+ in case of positivity for any HR type other than HPV16/31/33 or for LR types.

For the purpose of evaluating predictive ability of a triaging test, the crude (unadjusted) OR is the relevant comparison parameter. The adjusted OR analysis rather seeks to evaluate the etiology of the risk prediction, which is not relevant for the actual triaging. E.g., the strongly decreased OR for a LR positive test is essentially eliminated by adjusting for other HPV types. This indicates that a LR HPV infection does not in itself protect against CIN III+. Rather, a LR HPV positive test decreases the risk for CIN III+ simply because the test is not positive for a HR type. A weak risk predictive ability by age was removed by adjustment, suggesting that the predictive ability of age was explained by an age-dependent presence of a strong risk factor, e.g. an association of HPV16 with younger cases.

The sensitivity for HCII was close to 100% for CIN II+ and CIN III+, but a few women with CIN II+ were HCII-positive but not positive for any one of the HR HPV types. The small difference in sensitivity between HCII and PCR (counting only HR HPV-positivity as

positive) was due to the fact that a few cases of CIN II+ were positive for LR HPV types. Since we did not re-review the histopathological diagnoses, it is possible that some of the cases of CIN II+ could have been downgraded to CIN I on re-review. Some of the probes used in HCII are known to cross-react with several low-risk types [31] . The fact that some samples are falsely reported as HR-positive by HCII results in a lower cost efficiency when HPV-testing with HCII is used in triaging and unnecessary anxiety for the women who are presented with a false HR positive HPV-test.

For the present scientific study, we chose to use a non-commercial assay with open access availability. The test is based on the classical GP5+/6+ PCR system that has been validated in studies encompassing several hundred thousand women and is considered a reference test in the field [32].

Our study aimed to assess the predictive value of different HPV genotypes as such, not to evaluate a particular HPV assay. We recently performed a global proficiency study of 32 different HPV genotyping assays in terms of how well they accurately detect and genotype HPV [33]. As the assay used in the present study was found to be 100% proficient, our results are most likely generalizable to any proficient HPV genotyping assay.

HPV18 positivity did not confer any increased risk for CIN III+, which may appear surprising since HPV18 is the second most common HPV type in invasive cervical cancer [34]. HPV18 differs from HPV16 and its relatives HPV31/33 in that HPV18 is preferentially found in cervical adenocarcinoma. Cervical cytological screening is not very effective for preventing cervical adenocarcinoma and it is possible that the primary screening test (cytology) does not adequately identify the precursor lesions caused by HPV18. Furthermore, reports that have found increased CIN III+ risks for HPV18-positive women on long-term follow-up have found that these CIN III+ lesions appear more delayed after the HPV testing compared to the HPV16-associated CIN III+ lesions [17].

For clinical interpretation of the data obtained from HPV genotyping, it will be useful to provide an accurate ascertainment of the risks that are being conferred for each one of the so-called “high-risk” HPV types. HPV genotyping data from the ASCUS/LSIL triage study (ALTS) is consistent with the data in the present report, with HPV 16 being outstanding in associating with the highest risk for CIN III [35]Indeed, it should be noted that even though the present study provided full genotyping data for >1500 women, the statistical power is still

rather limited for the less common HPV types. Based on the data so far, it does appear that type-specific genotyping for HPV 16, 31 and 33 would be of interest and should be further explored. The risk for CIN III+ was not significantly increased among women with any other HR types detected than HPV 16, 31 or 33. HPV 16/31/33 were also the HPV types conferring highest risk for CIN II+ in a Swedish primary HPV-screening cohort[19].
Algorithms requiring detection also of the less than 20% of cases with other oncogenic HPV types than HPV16/31/33 would require referring almost twice as many women for colposcopy. It would be interesting to explore alternative management algorithms for women positive for the non HPV16/31/33 HR types (HPV 18, 35, 39, 45, 51, 52, 56, 58, 59, and 68), e.g. referring them for a repeat HPV test after 1 year.

Conceivable clinical management algorithms that use HPV genotyping data could e.g. be

a) Triaging using HPV 16 positivity. As the risk for CIN III+ is increased >5 times and most (57%) of CIN III+ cases are HPV16 positive, a conceivable strategy could be immediate referral and extended colposcopic investigation with multiple biopsies for HPV16-positive women, deferring HPV16-negative women to repeat HPV testing.
b) Triaging using HPV16/31/33 positivity, deferring women negative for these types to repeat HPV testing.

HPV 18 positivity merits special consideration. As the risk for CIN III+ at the time of testing, detected by conventional clinical investigation, is not increased, a standard immediate referral is not warranted. But the high risk on follow-up and the high risk for adenocarcinoma may warrant intensified follow-up of these women and may also warrant clinical investigation directed at detecting glandular lesions, such as endocervical brush cytology or endocervical curettage.
It should also be mentioned that HPV persistence is known to greatly increase the risk for CIN III+, for any oncogenic HPV type. Thus, if there is data from previous HPV genotyping demonstrating that the current positivity reflects persistence, or if genotyping of archival samples demonstrates persistence, immediate referral would most likely be advisable.

In summary, we have found that among women with ASCUS or LSIL in cytology, different HR HPV types have substantial differences in risk for the presence of CIN II+ with HPV16 being outstanding in terms of risk. Triaging algorithms that include HPV typing, at least for

HPV16 or for HPV16/31/33, appear to be promising for the further development of improved HPV-based triaging tests and algorithms.

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Table 1. HPV genotyping of 1595 women with ASCUS/LSIL in cytology, in comparison to subsequent CIN II+ or CIN III+ in histopathology.

HPV	Positive women N (%)	Patients with CINII+ (N)	Patients with CINIII+ (N)	Sensitivity CINII+ (%)	Sensitivity CINIII+ (%)	PPV CIN II+ (%)	PPV CIN III+ (%)
16	380 (23.8)	162	103	42.2	57.2	42.6	27.1
18	135 (8.5)	40	13	10.4	7.2	29.6	9.6
31	158 (9.9)	69	31	17.9	17.2	43.6	19.6
33	90 (5.6)	40	17	10.4	9.4	44.4	18.9
35	62 (3.9)	23	10	5.9	5.5	37.1	16.1
39	62 (3.9)	11	4	2.8	2.2	17.7	6.4
45	90 (5.6)	31	11	8.0	6.1	34.4	12.2
51	89 (5.6)	26	9	6.8	5.0	29.2	10.1
52	94 (5.9)	28	5	7.3	2.7	29.8	5.3
56	142 (8.9)	36	14	9.4	7.7	25.3	9.9
58	60 (3.8)	20	13	5.2	7.2	33.3	21.7
59	123 (7.7)	30	10	7.8	5.5	24.4	8.1
66	123 (7.7)	25	8	6.5	4.4	20.3	6.5
68	11 (0.7)	0	0	0	0	0	0
16/18	484 (30.3)	188	109	48.9	60.5	38.8	22.5
16/31/33	579	246	138	64.0	76.7	42.5	23.8

	(36.3)						
16/18/31/33	670 (42.0)	268	143	69.8	79.4	40.0	21.3
Other high risk types	473 (29.7)	97	30	25.2	16.7	20.5	6.3
Any high risk type	1148 (72.0)	365	173	95.0	96.1	31.8	15.1
Low risk types	140 (8.8)	8	2	2.0	1.1	5.7	1.4
HCII	1154 (72.4)	373	176	97.1	97.8	32.3	15.2
No triaging	1595 (100.0)	384	180	100.0	100.0	24.1	11.3

Table 2. Odds ratios (OR) of the risk for CIN II+ or CIN III+ for different HPV types.

Category	OR CIN II+ (95% CI)[a]		OR CIN III+ (95% CI)[a]	
	Crude	Adjusted	Crude	Adjusted
16	3.4 (2.65-4.37)	4.42 (3.32-5.89) [b]	5.55 (4.01-7.68)	6.22 (4.33-8.93) [b]
18	1.36 (0.92-2.0)	1.46 (0.95-2.26) [b]	0.82 (0.45-1.49)	0.82 (0.44-1.55) [b]
31	2.75 (1.96-3.85)	3.79 (2.61-5.52) [b]	2.10 (1.37-3.22)	2.83 (1.76-4.53) [b]
33	2.69 (1.74-4.14)	3.77 (2.34-6.07) [b]	1.91 (1.10-3.32)	2.70 (1.47-4.95) [b]
35	1.96 (1.15-3.33)	3.39 (1.91-6.02) [b]	1.37 (0.66-2.84)	2.23 (1.02-4.85) [b]
39	0.67 (0.34-1.29)	0.79 (0.39-1.6) [b]	0.53 (0.19-1.48)	0.65 (0.22-1.87) [b]
45	1.74 (1.10-2.73)	2.32 (1.40-3.83) [b]	1.24 (0.66-2.32)	1.68 (0.84-3.34) [b]
51	1.41 (0.88-2.27)	1.62 (0.96-2.73) [b]	0.92 (0.45-1.88)	0.99 (0.46-2.13) [b]
52	1.36 (0.86-2.14)	1.66 (1.01-2.73) [b]	0.42 (0.17-1.06)	0.50 (0.20-1.30) [b]
56	1.07 (0.72-1.59)	1.11 (0.71-1.72) [b]	0.84 (0.47-1.50)	0.93 (0.50-1.74) [b]

58	1.60 (0.92-2.77)	2.48 (1.36-4.53) [b]	0.55 (0.20-1.53)	0.76 (0.26-2.25) [b]
59	1.02 (0.67-1.57)	1.02 (0.63-1.63) [b]	0.68 (0.35-1.33)	0.82 (0.41-1.67) [b]
66	0.76 (0.48-1.21)	0.77 (0.46-1.28) [b]	0.53 (0.26-1.11)	0.58 (0.27-1.26) [b]
68	0	0 [b]	0	0 [b]
16/18	3.01 (2.37-3.83)	3.74 (2.83-4.93) [b]	4.28 (3.10-5.90)	4.52 (3.17-6.43) [b]
16/31/33	4.74 (3.71-6.05)	5.53 (4.18-7.31) [b]	7.38 (5.11-10.64)	7.52 (5.03-11.24) [b]
16/18/31/33	4.69 (3.65-6.02)	5.59 (4.18-7.48) [b]	6.63 (4.53-9.70)	6.75 (4.43-10.27) [b]
Other high risk types	0.76 (0.58-0.99)	2.66 (1.80-3.93) [c]	0.43 (0.28-0.65)	1.42 (0.82-2.47) [c]
Any high risk type	10.84 (6.65-17.65)	11.54 (6.65-20.05) [d]	10.84 (5.05-23.28)	11.19 (4.91-25.50) [d]
Low risk types	0.19 (0.09-0.41)	1.17 (0.47-2.87) [e]	0.13 (0.03-0.54)	0.74 (0.16-3.42) [e]
Any high and/or low risk types	12.08 (6.54-22.31)	12.20 (6.56-22.68) [f]	10.47 (4.27-25.68)	11.38 (4.60-28.18) [f]

Age (35+ versus <35)	0.70 (0.54-0.9)	1.05 (0.79-1.40)[g]	1.03 (0.66-1.27)	1.31 (0.78-1.35)[g]
Cytology at baseline (LSIL versus ASCUS)	1.09 (0.85-1.38)	0.90 (0.69-1.16)[h]	1.06 (0.76-1.47)	0.92 (0.66-1.30)[h]
HCII	20.38 (10.75-38.61)	13.16 (6.72-25.8)[i]	19.37 (7.14-52.54)	14.29 (5.06-40.41)[i]

a) ORs calculated using logistic regression.

b) Adjusted for co-infection with all other single HR types, LR types, age and baseline cytology.

c) Adjusted for co-infection with HPV 16, 18, 31, 33, and LR types, age and baseline cytology.

d) Adjusted for co-infection with LR types, age and baseline cytology.

e) Adjusted for co-infection with any HR types, age and baseline cytology.

f) Adjusted for age and baseline cytology.

g) Adjusted for co-infection with any HR and LR types and baseline cytology.

h) Adjusted for co-infection with any HR and LR types and age.

i) Adjusted for co-infection with all single PCR-positive HR types, LR types, age and baseline cytology.