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Possibly carcinogenic HPV subtypes are a cause of HSIL and negative clinical HPV tests – A European prospective single center study

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HIGHLIGHTS

- 9.5% of HSIL/AIS were missed by either Aptima or Cobas HPV test or both but were positive with HPV-PCR on lesional tissue.
- About 60% of missed HSIL were associated with possibly carcinogenic or low-risk HPV subtypes.
- 15% of missed HSIL were thin HSIL.
- 0.7% of HSIL were truly HPV-negative and associated with a somatic gene mutation or a single nucleotide variant.

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ABSTRACT

Objective. To correlate $p16^{ink4a}$ positive cervical precancers of 388 consecutive patients from a single European center with the preceding clinical HPV-DNA and HPV E6/E7 mRNA screening test.

Method. 374/388 patients had a HSIL (CIN 2/3) and 14/388 AIS (6 pure and 8 combined AIS/HSIL). Lesional tissues of HSIL/AIS with negative Cobas and/or Aptima HPV tests underwent HPV genotyping with CHIPRON HPV 3.5 LCD-array. Selected cases were subjected to a cancer hot spot analysis.

Results. The Aptima test missed 10/388 (2.6%) and the Cobas test seven of 388 (1.8%) precancers associated HPV-HR. Both HPV tests were negative in 20/374 precancers (5.3%; 17 HSIL/CIN3, two HSIL/CIN2, one AIS). Due to insufficient DNA four of 20 double negative cases (three HSIL, one AIS) were not genotyped. In the remaining cases, two of 20 (10%) HSIL genotyping detected HR-HPV subtypes. 10/20 (50%) HSIL were associated with possibly carcinogenic and low risk HPV (four x HPV73, three x HPV 53, one x HPV 82, one x HPV 67 and one x HPV 6), all of which are not included in both HPV tests. Two of 20 (10%) HSIL were negative with all HPV tests; one of these HSIL had a somatic PIK3CA gene mutation and the other had a single nucleotide variant in the APC gene. Three of 20 HSIL (15%) were thin HSIL (≤9 cell layers thick).

Conclusions. Possibly carcinogenic HPV subtypes not included in the clinical HPV tests may account for the small gap of missed HSIL in clinical HPV screening. True HPV negative HSIL are exceedingly rare. Expanding HPV testing to include more possibly carcinogenic HPV subtypes may further reduce cervical cancer.

1. Introduction

Cervical cancers arise via the precancerous lesions high-grade squamous intraepithelial lesion (HSIL) and adenocarcinoma in situ (AIS) after a transforming infection with human papilloma virus (HPV) high-risk (HR) genotypes [1]. The WHO divides HPV in carcinogenic HPV subtypes (group 1); probably carcinogenic HPV subtypes (group 2A); possibly carcinogenic HPV subtypes (group 2B); low risk HPV subtypes (group 3) and those not classified with respect to carcinogenic potential. Group 1 carcinogenic HPV high-risk subtypes, particular species HPV 16, HPV 18 and HPV 45 as main representative, are most commonly involved in cervical squamous and glandular carcinogenesis [2].

HPV-DNA-and HPV-E6/E7mRNA testing in primary cervical cancer screening is extremely reliable with a very high negative predictive value for detecting HSIL and AIS. Organized primary HPV-based cervical screening for young and middle-aged women becomes more and more established in many countries [3,4]. It is unclear however, how many HSIL/AIS will be missed with HPV-only screening. In the U.S.-based ATHENA study of a large screening population <2% of HSIL (CIN3) and AIS were negative by the Cobas HPV test. These lesions, however, tested positive with more sensitive HPV genotyping tests (Linear Array and Amplicor HPV testing) [5,6]. In European patient populations no

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Table 1
Characteristics of 388 patients with 374 $p16^{Ink4a}$ positive HSIL and 14 AIS and preoperative HPV DNA screening tests.

	Aptima negative Cobas negative	Aptima negative Cobas positive	Aptima positive Cobas negative
N (%)	20 (5.1%)	10 (2.6%)	7 (1.8%)
HSIL	19	10	7
AIS	1	0	0
HR-HPV genotyping of lesional tissue (SPF10)	2	7	3
LR-HPV genotyping of lesional tissue (SPF10)	2	0	0
Possibly carcinogenic HPV genotyping of lesional tissue (SPF10)	10	0	0
HPV negative genotyping of lesional tissue (SPF10)	0	0	0
Insufficient tissue (PCR)	4	3	4
Major colposcopic findings	12	5	4
Normal and minor abnormal colposcopic findings	8	5	3

systematic evaluations of HPV-DNA and/or HPV E6/E7 mRNA screening tests exist. Here we report our results from a prospective single center study re-examining HPV-DNA (Cobas-test) and HPV E6/E7 mRNA (Aptima test) negative HSIL/AIS of the cervix with the CHIPRON HPV 3.5 LCD-array.

2. Method

From January 2011 until August 2018 we identified consecutive 388 cone specimens (mean age 33 (18–74) years) with 374 $p16^{Ink4a}$ positive HSIL (Roche-mtm laboratories, Heidelberg, Germany) and 14 AIS and preoperative HPV DNA tests in liquid-based cytology specimens (Roche HPV test, Roche Molecular Systems, Pleasanton, USA) and mRNA Aptima HPV test (Hologic, USA). The Cobas HPV test reports out 12 pooled high-risk genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and simultaneously individual results for HPV 16 and HPV 18 in liquid-based specimens. The Aptima HPV test detects the mRNA of 14 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). H&E slides from cone specimens were re-reviewed by the study pathologists (SR and OR) with knowledge of patient age, HPV test results and p16 immunostaining. All cases with a falsely positive diagnosis of HSIL/AIS due to over interpretation of benign atypical changes or lesser-grade squamous intraepithelial lesions were excluded.

All patients were seen in the dysplasia unit of the Department of Obstetrics and Gynecology at Medical University of Graz. Specimens for both the Cobas and the Aptima HPV test were obtained in a standardized matter from the vial of liquid based cytology specimens. Indications for conization were histologically confirmed HSIL/AIS in colposcopic guided biopsy/endocervical curettage, persistence of high-grade abnormalities in cytology in the absence of positive histology and low-grade squamous intraepithelial lesion (LSIL) persistence >2 years (Tables 3–5). A negative clinical HPV test and/or a negative colposcopy did not influence excisional treatment.

All cases with a confirmed $p16^{Inka4a}$ overexpressing HSIL/AIS, but a negative Cobas and/or Aptima HPV test underwent HPV genotyping (CHIPRON GmbH, Berlin, Germany). HPV genotyping was performed on micro-dissected tissue of formalin fixed and paraffin embedded lesional tissue. The assay is based on two PCR reactions which are combined prior to hybridization into one array field. Both primer mixes are directed against highly conserved motifs within the viral L1 gene. Primer mix A generates fragments of approx. 450 bp in length (HPV type dependent), while primer mix B generates amplicon sizes of approx. 165 bp (HPV type dependent). SPF10 is a PCR based technique that detects a 65 bp region in the L1 open reading frame of the DNA of the virus. Combining both independent single PCRs prior to hybridization into one array field ensures the parallel and robust detection of all 32 HPV types at 5 target copies per reaction for HPV types 16, 18 and 31 and at 50 target copies for all other HPV types. It detects HPV-high risk subtypes 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59; HPV-low risk subtypes 6, 11; probably carcinogenic HPV subtype 68; possibly carcinogenic HPV subtypes 26, 53, 66, 67, 70, 73, 82, and the following not classified subtypes 42, 44, 54, 61, 62, 72, 81, 84, 90, 91. DNA was extracted on a Maxwell, MDx Research System (Promega, Fitchburg, Wisconsin, USA).

HSILs negative with all HPV tests were subjected to a cancer hotspot panel NGS analysis with the aim to evaluate somatic mutations for the top 50 most mutated cancer genes. NGS libraries were prepared using the AmpliSeq library kit 2.0 (Thermo Fisher Scientific) and the Ion Ampliseq Cancer Hotspot Panel V2 (Cat. Nr: 4475346) primer pool covering hotspot mutations in 50 genes implicated in cancer. Sequencing was performed on an Ion Proton benchtop sequencer (Thermo Fisher Scientific) to a length of 200 base pairs. Initial data analysis was done using the Ion Torrent Suite Software Plug-ins (Thermo Fisher Scientific, open source, GPL, https://github.com/iontorrent/). Briefly, this included base calling, alignment to the reference genome (HG19) using the TMAP mapper and variant calling by a modified diBayes approach taking into account the flow space information. Called variants were annotated using open source software ANNOVAR and SnpEff. All coding, nonsynonymous mutations were further evaluated and visually inspected in IGV (http://www.broadinstitute.org/igv/) and variant calls resulting from technical read errors or sequence effects were excluded from the analysis. Institutional review board approval was obtained (28-603ex.18 and 31-049 ex. 18/19).

3. Results

388 consecutive patients with $p16^{ink4a}$ positive HSIL (374 CIN 2/3) and AIS (14; six pure and eight combined AIS/HSIL) and preoperative clinical HPV testing were identified. 37/388 (9.5%) patients had one or two negative clinical HPV test results. The Aptima test failed to detect 10/388 (2.6%) and the Cobas test missed seven of 388 (1.8%) precancerous lesions. All precancers with a single negative screening test were picked up by the CHIPRON HPV 3.5 LCD-array. All precancers with a single and double negative test result were subjected to HPV genotyping of lesional tissue. A total of eight of 37 (22%) negative precancers could not be genotyped due to insufficient DNA (Table 1).

Table 2
Characteristics of 20/388 patients with negative results in Aptima and Cobas HPV-test.

HPV classification (SPF10)	HPV type	N (%)
Possibly carcinogenic	73, 53, 67, 82	10 (50%)
High risk	52, 58[a]	2 (10%)
Low risk	6	2 (10%)
Negative	–	2 (10%)
Insufficient tissue (PCR)	–	4 (20%)

[a] One lesion with coinfection of HPV 90.

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Table 3
Characteristic of 20 of 388 patients with HSIL/AIS and negative results in the Aptima and Cobas HPV test.

Age	Indication for conization	Colposcopic findings	SPF10 HPV genotyping on lesional tissue	HPV classification	Histology cone specimen
51	HSIL (CIN 3)	Major	52	High-risk	HSIL (CIN 3)
28	HSIL (CIN 3)	Major	82	Possibly carcinogenic	HSIL (CIN 3)
27	HSIL (CIN 3)	Major	82	Possibly carcinogenic	HSIL (CIN 3)
34	HSIL (CIN 3)	Minor	53	Possibly carcinogenic	HSIL (CIN 3) Thin variant
57	HSIL (CIN 3)	Major	–	Negative	HSIL (CIN 3)
54	HSIL (CIN 2)	Normal	73	Possibly carcinogenic	HSIL (CIN 3) Thin variant
53	HSIL (CIN 3)	Normal	53	Possibly carcinogenic	HSIL (CIN 3)
39	HSIL (CIN 3)	Major	73	Possibly carcinogenic	HSIL (CIN 3)
37	LSIL persistence	Miscellaneous	6	Low-risk	HSIL (CIN 3)
28	HSIL (CIN 3)	Minor	53	Possibly carcinogenic	HSIL (CIN 2)
36	HSIL (CIN 3)	Major	73	Possibly carcinogenic	HSIL (CIN 3)
38	HSIL (CIN 3)	Major	58, 90	High risk; not classified	HSIL (CIN 2)
42	Persistent abnormal Cytology	Normal	–	Negative	HSIL (CIN 3)
38	HSIL (CIN 3)	Major	73	Possibly carcinogenic	HSIL (CIN 3)
30	HSIL (CIN 3)	Major	Insufficient tissue		HSIL (CIN 3)
28	HSIL (CIN 3)	Minor	Insufficient tissue		HSIL (CIN 3)
33	HSIL (CIN 3)	Major	67	Possibly carcinogenic	HSIL (CIN 3)
35	HSIL (CIN 3)	Major	Insufficient tissue		HSIL (CIN 3)
20	HSIL (CIN 2) persistence	Minor	6	Low risk	HSIL(CIN 3) Thin variant
21	AIS	Major	Insufficient tissue	–	AIS

3.1. Double negative HSIL and AIS (Tables 2 and 3)

20/388 patients with precancers (5.1%; 19 HSIL, one pure AIS) had a negative Cobas and Aptima HPV test. Four of 20 (20%) double negative HSIL could not be further genotyped due to insufficient DNA amounts (two HSIL/CIN 3, one HSIL/CIN 2, one AIS). HPV-genotyping of lesional tissues identified HR-HPV subtypes in 2/20 (10%) HSIL with HPV 52 in one HSIL/CIN3 and HPV 58 & HPV 90 in one HSIL/CIN 2. HPV genotyping of 10/20 HSIL detected HPV subtypes currently classified as possibly carcinogenic (four x HPV73, three x HPV53, two x HPV82, one x HPV67). Three of 10 HSIL with possibly carcinogenic HPV subtypes were thin HSIL with ≤9 cell layers thickness. Two of 20 HSIL (10%) were associated with low-risk HPV 6. Two of 20 HSIL/CIN 3 (10%) were negative with all HPV tests (Cobas-test, Aptima test, genotyping with CHIPRON HPV 3.5 LCD-array). These two cases were further subjected to cancer hotspot panel analysis revealing a somatic gene mutation in the PIK3CA gene in one patient and a rare genomic single nucleotide variant of APC regulator of WNT signaling pathway gene (variant: NM_000038.6(APC): c.4073C>T (p.Ala1358Val) location 5q22.2) classified as "unknown significance" in the ClinVar database (Variation ID: 181805) in the other patient.

Indication for excisional treatment was based on histological diagnosis of HSIL/AIS in 18/20 patients, persistence of HSIL/CIN 2 in one patient and persistent abnormal cytology in one patient. Preoperative colposcopy showed major abnormal colposcopic findings in 13/20 women whereas the other seven patients had minor abnormal, miscellaneous or normal colposcopic findings.

3.2. Aptima test negative HSIL in patients with positive Cobas test results (Table 4)

10/388 patients (2.6%) or 10/374 HSIL (2.7%) had a negative Aptima test, but the Cobas test identified HR HPV in 9/10 HSIL (three x HPV 16, one x HPV 18 and six x HPV "other"). Genotyping of lesional tissue of HSIL associated with "other" HPV genotypes demonstrated HPV 52 in two patients, and one patient each with a coinfection of HPV 52 and HPV 58, HPV 58 and HPV 90, and HPV 16, 53 and 81. Three HSIL (one x CIN 2, two x CIN 3) could not be further classified due to insufficient DNA amounts. Indication for excisional treatment was based on histological diagnosis of HSIL in 8/10 patients, persistence of LSIL in one patient and persistent abnormal cytology in one patient. Preoperative colposcopy showed major abnormal colposcopic findings in 5/10 women whereas the other five patients only had minor abnormal colposcopic findings.

3.3. Cobas test negative HSIL in patients with positive Aptima test results (Table 5)

Seven of 388 patients (1.8%) with HSIL had negative results in the Cobas test but were positive for the Aptima test. HPV genotyping of lesional tissue identified high-risk HPV 16 (two of seven), HPV 52

Table 4
Characteristic of 10 of 388 patients with HSIL/AIS and negative Aptima test results but positive findings in Cobas HPV test.

Age	Indication for conization	Colposcopic findings	HPV test Cobas	SPF10 HPV genotyping on lesional tissue	Histology cone specimen
26	LSIL persistence	Minor	18	18	HSIL (CIN 3)
30	HSIL (CIN 3)	Major	Other	58, 90	HSIL (CIN 3)
23	HSIL (CIN 2) persistence	Major	Other	52	HSIL (CIN 2)
35	HSIL (CIN 2) persistence	Minor	Other	Insufficient tissue	HSIL (CIN 2)
34	AIS in cytology	Minor	16	Insufficient tissue	HSIL (CIN 3)
23	HSIL (CIN 3)	Major	16	16,53,81	HSIL (CIN 3)
45	HSIL (CIN 3)	Minor	Other	Insufficient tissue	HSIL (CIN 3)
26	HSIL (CIN 2) persistence	Minor	Other	52	HSIL (CIN 2)
21	HSIL (CIN 2) persistence	Major	Other	52,58	HSIL (CIN 3)
57	HSIL (CIN 3)	Major	16	16	HSIL (CIN 3)

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Table 5
Characteristic of seven of 388 patients with HSIL/AIS and Cobas test negative results but positive findings in HPV Aptima test.

Age	Indication for conization	Colposcopic findings	SPF10 HPV genotyping on lesional tissue	HPV classification	Histology cone specimen
35	HSIL (CIN 3)	Major	16	High risk	HSIL (CIN 3)
49	HSIL (CIN 3)	Normal	58	High-risk	HSIL (CIN 3)
41	Persistence abnormal cytology	Minor	Insufficient tissue		HSIL (CIN 3)
31	HSIL (CIN 3)	Major	16	High-risk	HSIL (CIN 3)
44	HSIL (CIN 3)	Major	Insufficient tissue		HSIL (CIN 3)
36	HSIL (CIN 3)	Major	52	High-risk	HSIL (CIN 3)
35	LSIL persistence	Minor	Insufficient tissue		HSIL (CIN3)

(one of seven), and HPV 58 (one of seven). HPV Genotyping of in three HSIL/CIN 3 cases was not possible due to insufficient DNA amount. Indication for excisional treatment was histological diagnosis of HSIL in five patients, LSIL persistence in one patient and persistent abnormal cytology in one patient. Preoperative colposcopy showed major abnormal colposcopic findings in four women and minor abnormal colposcopic findings three patients.

4. Discussion

The main findings of this prospective study are that a small percentage of HSIL were missed by presently commercially available clinical HPV tests. About 60% of double negative/missed HSIL were associated with possibly carcinogenic HPV subtypes or low-risk HPV subtypes. 15% of missed HSIL were thin HSIL with only normal or minor abnormal colposcopic findings, and 0.7% HSIL/CIN3 were truly HPV-negative HSIL.

The strengths of our study are a standardized specimen collection, a histopathological review by two gynecopathologists to exclude misclassifications and confirmation of clinical test results by HPV genotyping of formalin-fixed paraffin-embedded lesional tissue. A weakness in this study relates to the fact that some small lesions could not be further classified due to insufficient DNA amounts.

Clinical HPV tests have been developed under the assumption that HSIL/AIS should not occur in woman with a negative test result. When this occurs the following scenarios can explain this situation. Firstly, it is a truly HPV negative HSIL/AIS, Secondly, lesions are induced by non-high risk HPV genotypes not included in these tests. Thirdly, the false negative HPV test is due to of sampling artefact or insufficient DNA amount/quality. A false positive histological diagnosis of HSIL/AIS due to over interpretation of benign atypical changes or lesser-grade SIL. These cases, however, were excluded in this study.

In our study the most frequent reason for Cobas and Aptima HPV negative tests was HSIL induced by possibly carcinogenic HPV subtypes that are not included in the presently commercially available clinical HPV tests. In our European patient population HPV 73 and HPV 53 were the most commonly detected non-high risk HPV subtypes, followed by HPV 82 and HPV 67. HPV 82 und HPV 73 were also the most relevant subtypes in false-negative HPV tests in the ATEHNA trial [5]. Eight non-high-risk HPV types (HPV 26, 53, 66, 67, 68, 70, 73 and 82) have been identified consistently as single HPV infections in about 3% of cervical cancer tissues [7].

Single LR-HPV infections have only a minor impact on the overall burden of HPV-related cancers. Nevertheless, it is well known that a very small portion of HSIL and invasive cervical cancers (0.4% of cases) is associated with low-risk HPV [8–10]. Both the Athena trial [5] and results from our European patient population confirm these numbers.

Interestingly, three of 20 patients (15%) with double negative HPV tests had a thin HSIL. These are small lesions that are ≤9 cells thick. They develop in early metaplastic squamous epithelium of the transformation zone without anteceding LSIL [11,12]. Little is known about the natural course of these lesions, if they develop into a thick HSIL over time and if they have a risk for invasion [8]. In our series all three thin HSIL were associated with non-HR-HPV subtypes. Colposcopy was normal or showed minor abnormal findings.

Among the 14 patients with AIS 93% AIS were detected by the Cobas and Aptima test. The only AIS with double negative tests could not be further classified due to insufficient DNA amount. These results are in line with those of a multination European epidemiological study showing a 94% HPV-positivity rate in of AIS [13].

HPV-negative HSIL/CIN 3 is poorly documented. The Athena trial could not identify a single case of true HPV-negative HSIL/AIS [5]. We report two cases of full thickness lesions histologically indistinguishable from HSIL/CIN3 that were negative with all HPV tests (Cobas test, Aptima test und genotyping with CHIPRON HPV 3.5 LCD-array). One of these showed a somatic gene mutation in the PIK3CA gene. PIK3CA gene mutations have been described in 30% of invasive squamous cell cancer but in <3% of HSIL/CIN 3 [14–16]. The other case had a rare genomic single nucleotide variant of APC regulator of WNT signaling pathway gene (variant: NM_000038.6(APC): c.4073C>T (p.Ala1358Val) location 5q22.2) of "unknown significance" [17].

Our study indicates that nearly every tenth woman with HSIL would be missed with HPV-only screening with presently available HPV tests. Missed HSIL include those induced by HPV subtypes not included in the clinical HPV-tests, in particular, potentially and possibly carcinogenic HPV subtypes, LR-HPV-induced HSIL and truly HPV-negative HSIL. Incorporating probably and possibly carcinogenic HPV subtypes into screening tests would enhance the clinical usefulness of these tests, in particular with respect to exit screening tests.

CRediT authorship contribution statement

Olaf Reich: Conceptualization, Writing - original draft, Writing - review & editing, Formal analysis, Investigation.**Sigrid Regauer:**Conceptualization, Writing - original draft, Writing - review & editing, Formal analysis, Investigation.**Karl Kashofer:**Investigation, Formal analysis.

Declaration of competing interest

The authors declare no conflict of interests.

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