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High-grade vulvar intraepithelial neoplasia: comprehensive characterization and long-term vulvar carcinoma risk

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High-grade vulvar intraepithelial neoplasia: comprehensive characterization and long-term vulvar carcinoma risk

Aims: Adequate diagnosis of human papillomavirus (HPV)-associated high-grade squamous intraepithelial lesion (HSIL) and HPV-independent vulvar intraepithelial neoplasia (VIN) is essential but can be challenging. We comprehensively characterized a large population-based series of vulvar lesions, originally reported as high-grade VIN, and assessed the cancer risk.

Methods and results: Baseline high-grade VIN of 751 patients were categorized by histopathological reassessment, integrating the results of immunohistochemistry ($p16^{INK4a}$, p53, Ki-67) and HPV DNA testing. Integrated analyses resulted in 88.4% HPV-associated lesions (77.0% HSIL, 10.9% low-grade SIL [LSIL], and 0.4% vulvar squamous cell carcinoma [VSCC]), 10.9% HPV-independent lesions (6.1% HPV-independent VIN, 4.7% nondysplastic lesions, and 0.1% VSCC) and 1.1% inconclusive lesions. HSIL demonstrated $p16^{INK4a}$ block-positivity in 99.0%, increased Ki-67 in ≥2/3rd of the epithelium in 93.6%, and HPV positivity in 99.6%. In HSIL, a p53 wildtype mid-epithelial staining pattern was common (51.6%) while this was not observed in HPV-independent lesions. HPV-independent VIN harboured mutant p53 patterns in 65.2% and showed a wide morphological spectrum, ranging from differentiated to nondifferentiated ('HPV-associated-like', in 41.3%). Kaplan–Meier analyses showed a 10-year cancer risk of 8.0% in HPV-associated HSIL, 67.4% in HPV-independent VIN/p53mutant, and 27.8% in HPV-independent VIN/p53wildtype. Strikingly, the 10-year cancer risk was 73.3% in HPV-independent VIN with nondifferentiated ('HPV-associated-like') morphology.

Conclusion: Immunohistochemistry by $p16^{INK4a}$ and p53 is highly recommended for optimal categorization into HPV-associated and HPV-independent VIN, which is of utmost importance given the different cancer risk. The high cancer risk of HPV-independent VIN underscores the need for surgical treatment and close follow-up, especially in case of a p53 mutant pattern and/or nondifferentiated morphology.

Keywords: cancer risk, dVIN, HPV, HSIL, vulvar intraepithelial neoplasia

Introduction

Vulvar intraepithelial neoplasia (VIN), the precursor of vulvar squamous cell carcinoma (VSCC), is categorized into HPV-associated high-grade squamous

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intraepithelial lesion (HSIL) and low-grade SIL (LSIL), and HPV-independent VIN.[1] HPV-associated SIL occurs mainly in younger women and is treated by imiquimod, excision, or laserevaporiazation.[2,3] HPV-independent VIN, often referred to as differentiated VIN (dVIN), occurs mainly in older women in a background of lichen sclerosus (LS) or lichen planus and is treated by excision.[2,4] HPV-independent VIN often has a history of vulvar cancer or is diagnosed adjacent to cancer.[5,6]

In contrast to HPV-associated SIL, HPV-independent VIN shows a wide spectrum of clinical and histomorphologic features, some overlapping with reactive/nondysplastic dermatoses.[7–9] Given the high cancer risk in HPV-independent VIN, misclassified lesions can have serious clinical consequences. For optimal typing and grading of VIN, a few immunohistochemical (IHC) markers have been established. The Lower Anogenital Squamous Terminology (LAST) recommends the use of $p16^{INK4a}$ to differentiate between HSIL and LSIL.[10] To diagnose HPV-independent VIN, the use of p53 IHC can be helpful. However, caution is needed, as approximately one-third of HPV-independent VIN lacks a mutant p53 pattern, while 'mutant-like' patterns, such as wildtype staining with markedly reduced staining intensity mimicking mutant 'null' staining, and wildtype mid-epithelial staining with basal sparing, mimicking mutant positive staining, can be seen in HPV-associated lesions.[11,12]

The aim of this study was to categorize 751 vulvar lesions originally diagnosed as high-grade VIN (hg-VIN) into HPV-associated or HPV-independent categories by integrated analyses of histopathologic review, IHC results, and HPV DNA testing, and to determine the cancer risk for different subgroups of hg-VIN.

Materials and methods

STUDY POPULATION

From a population-based historical cohort, a total of 894 patients diagnosed with hg-VIN (originally 884 HSIL and 12 HPV-independent VIN) between 1991 and 2011 were identified, as described previously.[6] Patients with prior or concurrent (i.e. within 3 months) VSCC were not included. Formalin-fixed, paraffin-embedded tissue blocks of the baseline hg-VIN were retrieved. In order to determine progression to cancer, follow-up data were collected up to 2020, as previously described.[6]

This study was approved by the local Medical Ethics Committee of Amsterdam UMC, location VUmc. Informed consent was not required.

CATEGORIZATION OF VULVAR LESIONS

Categorization was based on histopathological assessment by two pathologists (M.C.G.B., N.B.T.) with integrated analyses of IHC and HPV results. Vulvar lesions were categorized as HPV-associated (VSCC, HSIL, LSIL) or HPV-independent (VSCC, HPV-independent VIN, nondysplastic lesions, including LS, reactive lesions, and other nondysplastic dermatoses). For HPV-independent VIN, differentiated and nondifferentiated ('HPV-associated-like') morphology was recorded. Nondifferentiated ('HPV-associated-like') morphology included lesions mimicking HSIL or LSIL, as described by Rakislova *et al.*[13] In short, nondifferentiated ('HPV-associated-like') morphology represented all morphologies without epithelial differentiation characterizing 'classical' dVIN. This included both basaloid morphology, consisting of full-thickness epithelial atypia and high nuclear-to-cytoplasmic ratio, and the remainder of nondifferentiated morphologies, mainly comprising papillary epithelium (whether or not inverted) with elongated, bulbous rete ridges, moderate to marked pleomorphism, and koilocytic-like changes.[13,14] Adjacent to areas of 'HPV-associated-like' HPV-independent VIN', more typical areas of dVIN could be seen.

TISSUE PROCESSING

Details of tissue processing, IHC of $p16^{INK4a}$, p53, and Ki-67, DNA isolation, and HPV DNA testing are described in Data S1.

IMMUNOHISTOCHEMICAL STAINING PATTERNS OF $P16^{INK4A}$, P53, AND KI-67

Examples of the IHC staining patterns are presented in Figure 1. $P16^{INK4a}$ staining was scored as negative (absent or patchy) or block (diffuse) positive (≤1/3, ≤2/3, >2/3).[15] p53 staining was scored as wildtype (scattered or mid-epithelial with basal sparing) or mutant (nuclear positive including basal aberrant and parabasal/diffuse aberrant, null or cytoplasmic positive). A mutant positive staining pattern included the earlier described categories of 'basal overexpression' (i.e. uniformly strong nuclear staining in at least 80% of the basal cells without significant parabasal staining) and 'parabasal/diffuse overexpression' (i.e. uniformly strong nuclear staining of both the basal and the parabasal cells).[16,17] Ki-67 staining was scored as not increased (a few positive parabasal nuclei) or increased (≤1/3, ≤2/3, >2/3).[15] In addition, there was a so-called 'viral' Ki-67 staining pattern

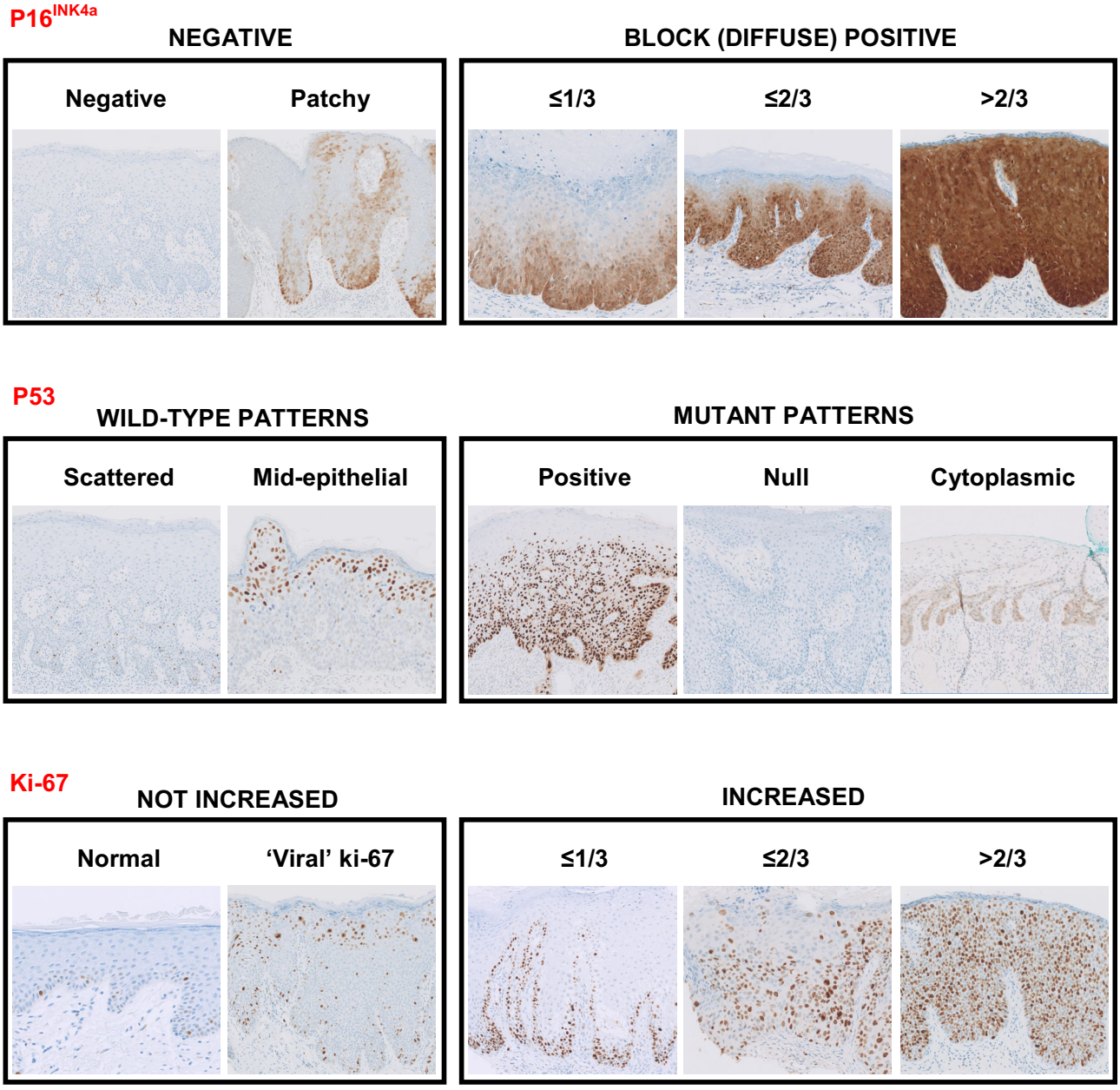

Figure 1. Representative examples of p16[INK4a], p53, and Ki-67 immunohistochemical staining patterns. The p16, p53, and the increased Ki-67 staining patterns haven been described before.[15–17] The p53 mutant positive pattern includes the earlier described patterns of 'basal overexpression' and 'parabasal/diffuse overexpression'.[16,17]

when there was increased staining in the upper layers with less or no increased staining in the lower layers.

HUMAN PAPILLOMAVIRUS (HPV) GENOTYPING

HPV DNA testing was performed for high-risk (hr) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 ('possibly carcinogenic'), and 68 ('probable carcinogenic').[18] In case a lesion was assumed to be HPV-associated after histopathological assessment and tested negative for hr-HPV DNA, additional testing for low-risk (lr) types 6, 11, 32, 39, 40, 42, 43, 44, 54, 55, 57, 61, 71, 72, 81, 83, 84, and 86, and 'possible high-risk' types 26, 30, 34, 53, 67, 69, 70, 73, 82, and 85, was performed.[19,20]

STATISTICAL ANALYSIS

Cumulative VSCC incidence was determined in three subgroups: HPV-associated HSIL, p53 mutant HPV-independent VIN (HPV-independent VIN/p53mut), and p53 wildtype HPV-independent VIN (HPV-independent/p53wt). Additionally, stratified analyses for the morphological subtype of HPV-independent VIN, i.e. differentiated or nondifferentiated ('HPV-associated-like'), were done. Cumulative VSCC incidence was calculated from baseline hg-VIN to the date of VSCC with the Kaplan–Meier adjusting for censoring with the 95% confidence interval (CI).[6] Details on censoring are described in Data S1.[21] Cancer risk differences were evaluated by log-rank tests. The level of statistical significance was set at 0.05. Statistical analysis was performed using IBM SPSS Statistics software for Windows v. 28.0 (IBM, Armonk, NY, USA) and graphs were produced in GraphPad Prism 9 (San Diego, CA, USA).

Results

STUDY POPULATION

From 791/894 (88.5%) patients, tissue blocks of baseline hg-VIN were retrieved. Subsequently, 40 cases were excluded due to insufficient tissue, resulting in 751 (84.0%) vulvar lesions. Median follow-up time was 17.3 years (range: 8.3–35.4).

CATEGORIZATION OF HPV-ASSOCIATED AND HPV-INDEPENDENT LESIONS

Final categorization in relation to the original diagnoses and age is shown in Table 1. Most lesions were HPV-associated (88.4%) and were categorized as HSIL (77.0%) or LSIL (10.9%). Three cases (0.4%) had the presence of microinvasive disease and were categorized as VSCC. A minority of lesions (10.9%) was HPV-independent and categorized as HPV-independent VIN (6.1%), nondysplastic lesions (4.7%), or VSCC (0.1%). The diagnosis was inconclusive for 1.1% of lesions, mainly because no distinction between HPV-associated and HPV-independent could be made. Patients with HPV-associated lesions had a lower median age compared to patients with HPV-independent lesions ($P < 0.001$), but a wide range was observed.

Categorization in relation to the IHC and HPV genotyping results is depicted in Tables 2 and 3 and Table S1.

HPV-ASSOCIATED HIGH-GRADE SQUAMOUS INTRAEPITHELIAL LESIONS (HSIL)

HSIL was usually easy to diagnose by haematoxylin and eosin (H&E) assessment only (Figure 2A–D). In HSIL, block-positive $p16^{INK4a}$ was observed in 99.0%, hr-HPV was detected in 99.3% of HPV positive HSIL, and HPV16 was present in 81.3% of HPV positive HSIL. Six (1.0%) HSIL were $p16^{INK4a}$ negative

Table 1. Categorization of vulvar lesions after reassessment in relation to the original diagnosis and age at baseline

	Original diagnosis			Age, years
	HSIL	DVIN	Total (%)	Median (range)
	743	8	751 (100)	45.0 (16–92)
Final categorization				
HPV-associated	663	1	664 (88.4)	44.0 (17–91)
HSIL	578	0	578 (77.0)	45.0 (17–90)
LSIL	81	1	82 (10.9)	39.0 (19–91)
VSCC	3	0	3 (0.4)	39.0 (37–48)
HPV-independent	75	7	82 (10.9)	67.0 (16–92)
HPV-independent VIN	39	7	46 (6.1)	72.0 (35–92)
Nondysplastic	35	0	35 (4.7)	58.0 (16–79)
VSCC	1	0	1 (0.1)	67.0 (NA)
Inconclusive	6	0	6 (1.1)	75.0 (46–88)

Abbreviations: dVIN, Differentiated vulvar intraepithelial neoplasia; HPV, Human papillomavirus; HSIL, High-grade squamous intraepithelial lesion; LSIL, Low-grade squamous intraepithelial lesion; NA, Not applicable; VSCC, Vulvar squamous cell carcinoma.

Table 2. Immunohistochemical staining patterns of p16^{INK4a}, p53, and Ki-67 in human papillomavirus (HPV)-associated and HPV-independent vulvar lesions

	HPV-associated		HPV-independent	
	HSIL+	LSIL	HPV-independent VIN+	Nondysplastic
P16^{INK4a}				
Negative				
Negative	1 (0.2)	23 (28.0)	32 (68.1)	26 (74.3)
Patchy	5 (0.9)	34 (41.5)	14 (29.8)	9 (25.7)
Block positive				
≤1/3	50 (8.6)	15 (18.3)	0 (0)	0 (0)
≤2/3	289 (49.8)	10 (12.2)	0 (0)	0 (0)
>2/3	235 (40.5)	0 (0)	1 (2.1)	0 (0)
p53				
Wildtype				
Scattered	278 (48.0)	61 (74.4)	16 (34.8)	35 (100)
Mid-epithelial	300 (51.8)	21 (25.6)	0 (0)	0 (0)
Mutant pattern				
Positive	1 (0.2)	0 (0)	19 (41.3)	0 (0)
Null	0 (0)	0 (0)	11 (23.9)	0 (0)
Cytoplasmic	0 (0)	0 (0)	0 (0)	0 (0)
Ki-67				
Not increased				
Normal	2 (0.3)	20 (24.4)	6 (12.8)	19 (54.3)
'Viral' Ki-67	38 (6.6)	32 (39.0)	0 (0)	0 (0)
Increased				
≤1/3	36 (6.2)	55 (67.1)	36 (76.6)	14 (40.0)
≤2/3	317 (54.7)	7 (8.5)	4 (8.5)	2 (5.7)
>2/3	224 (38.7)	0 (0)	1 (2.1)	0 (0)

In four lesions, one or more stains could not be assessed (1× p16^{INK4a}, 4× p53, and 2× Ki-67).
HSIL+, High-grade squamous intraepithelial lesion, including three HPV-associated vulvar squamous cell carcinomas; HPV-independent VIN+, HPV-independent vulvar intraepithelial neoplasia, including one HPV-independent vulvar squamous cell carcinoma; LSIL, Low-grade squamous intraepithelial lesion.

(Figure 2E–H), all positive for HPV16, with wildtype p53 staining and with HSIL morphology, supported by increased Ki-67 in ≥2/3 of the epithelium. Six HSIL were negative for hr-HPV, all block-positive for p16^{INK4a}, with wildtype p53 staining and 4/6 positive for lr-HPV or 'possible hr'-HPV (type 6, 6/34, 26, and 83). Increased Ki-67 in ≥2/3rd of the epithelium was encountered in 93.6% of HSIL. Many HSIL showed a reduced staining intensity of p53, mimicking a p53 mutant null pattern (Figure 3A–D). p53 mid-epithelial staining with sparing of the basal cell layer was observed in 51.6% of HSIL (Figure 3E–H). One (0.2%) HSIL showed mutant positive p53 staining in 30% of the lesion. This lesion showed obvious HSIL morphology, block-positive p16^{INK4a}, and harboured hr-HPV.

Table 3. High-risk and low-risk human papillomavirus (HPV) genotype distribution per disease category.

	HPV-associated		HPV-independent	
	HSIL+	LSIL	HPV-independent VIN+	Nondysplastic
Overall HPV positive	557/559 (99.6)	56/62 (90.3)	5/34 (14.7)	0/11 (0)
High-risk HPV positive	553 (99.3)	43 (76.8)	4 (80.0)	0 (0.0)
Single high-risk HPV type	535 (96.1)	42 (75.0)	4 (80.0)	0 (0.0)
Multiple high-risk HPV types	18 (3.2)	1 (1.8)	0 (0.0)	0 (0.0)
High-risk HPV genotype 16/18	479 (86.0)	32 (57.1)	3 (60.0)	0 (0.0)
Type 16	453 (81.3)	30 (53.6)	3 (60.0)	0 (0.0)
Type 18	27 (4.8)	3 (5.4)	0 (0.0)	0 (0.0)
High-risk HPV genotype non-16/18	89 (16.0)	11 (19.6)	1 (20.0)	0 (0.0)
Type 31	2 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)
Type 33	41 (7.4)	2 (3.6)	0 (0.0)	0 (0.0)
Type 35	1 (0.2)	1 (1.8)	0 (0.0)	0 (0.0)
Type 45	5 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)
Type 51	4 (0.7)	1 (1.8)	0 (0.0)	0 (0.0)
Type 52	1 (0.2)	1 (1.8)	0 (0.0)	0 (0.0)
Type 56	2 (0.4)	1 (1.8)	0 (0.0)	0 (0.0)
Type 59	2 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)
Type 66[a]	2 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)
Type undetermined (variant X)	9 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)
Type non-16/18, not further specified[b]	21 (3.8)	5 (8.9)	1 (20.0)	0 (0.0)
Tested for additional HPV types	8/559 (1.4)	16/62 (25.8)	15/34 (44.1)	2/11 (18.2)
Low-risk HPV positive	4 (0.7)	13 (23.2)	1 (20.0)	0 (0)
Single low-risk HPV type	3 (0.5)	12 (21.4)	1 (20.0)	0 (0)
Multiple low-risk HPV types	1 (0.2)	1 (1.8)	0 (0)	0 (0)
Low-risk HPV genotype				
Type 6	2 (0.4)	11 (19.6)	0 (0)	0 (0)
Type 11	0 (0.0)	0 (0.0)	1 (20.0)	0 (0)
Type 26[a]	1 (0.2)	0 (0.0)	0 (0)	0 (0)
Type 34[a]	1 (0.2)	0 (0.0)	0 (0)	0 (0)
Type 42	0 (0.0)	3 (5.4)	0 (0)	0 (0)
Type 83	1 (0.2)	0 (0.0)	0 (0)	0 (0)

Type-specific positivity includes those contributed by multiple infections.
HSIL+, High-grade squamous intraepithelial lesion, including three HPV-associated vulvar squamous cell carcinomas; LSIL, Low-grade squamous intraepithelial lesion; HPV-independent VIN+, HPV-independent VIN, including one HPV-independent vulvar squamous cell carcinoma.
[a]IARC (International Agency for Research on Cancer) Group 2b ('possibly carcinogenic').[17]
[b]'High-risk HPV Type non-16/18, not further specified' was used for cases that could not be subtyped due to insufficient DNA.

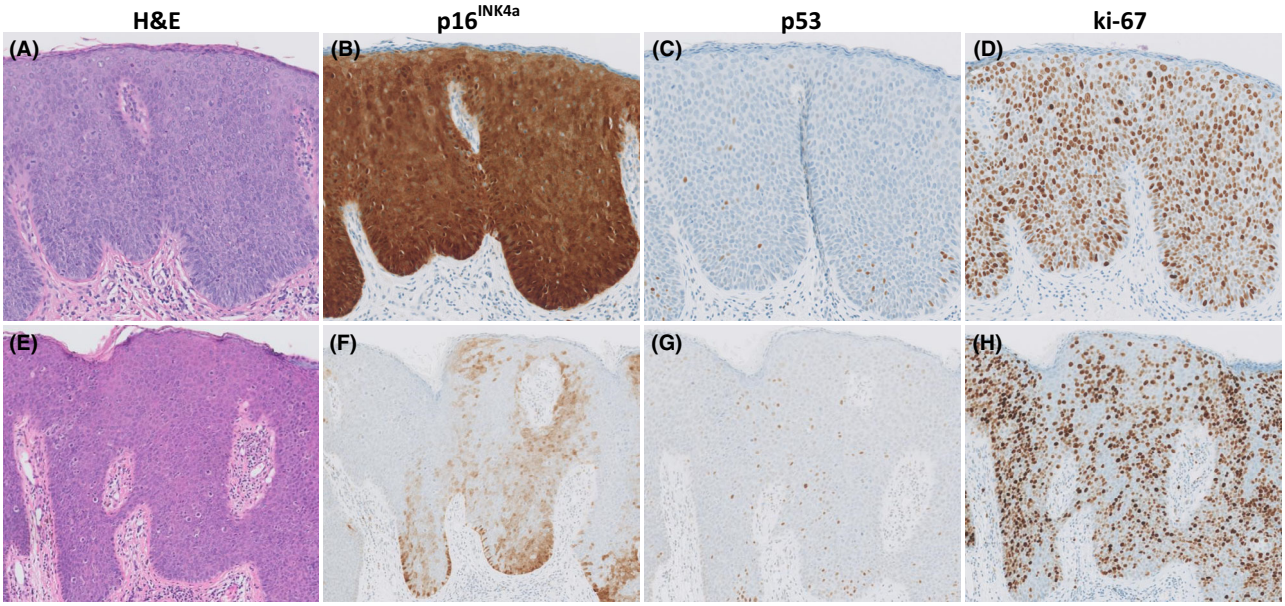

Figure 2. HPV-associated vulvar high-grade squamous intraepithelial lesion (HSIL). (A–D) Representative example of 'classical' HSIL with block-positive p16^{INK4a}, wildtype, scattered p53 staining, and full-thickness increased Ki-67. (E–H) HSIL with patchy (negative)p16^{INK4a}, HSIL morphology, wildtype, scattered p53, and full-thickness increased Ki-67.

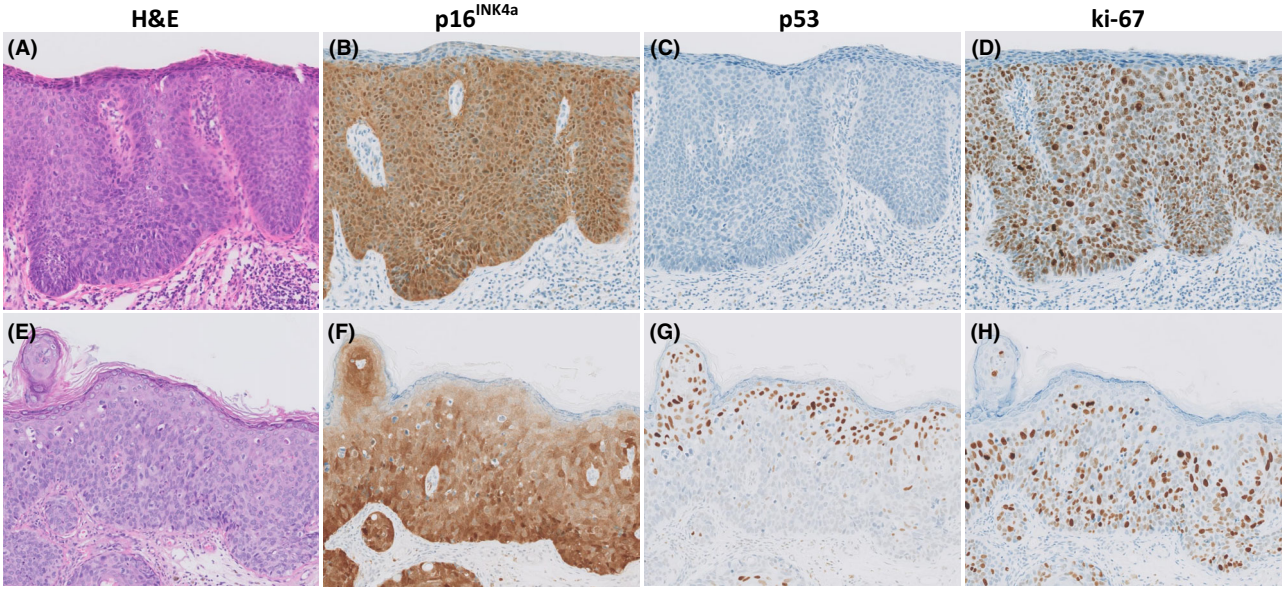

Figure 3. HPV-associated vulvar high-grade squamous intraepithelial lesion (HSIL). (A–D) HSIL with wildtype, reduced p53 staining, mimicking a mutant null pattern. (E–H) HSIL with wildtype p53 mid-epithelial staining with sparing of the basal cell layer, which can mimic mutant positive staining, and with positive p16^{INK4a}.

HPV-ASSOCIATED LOW-GRADE SQUAMOUS INTRAEPITHELIAL LESIONS (LSIL)

Examples of LSIL are shown in Figure 4A–D. In LSIL, p16^{INK4a} block-positivity was found in 30.5%. Viral lesions without dysplasia (n = 18) were all p16^{INK4a}-negative. Overall, HPV and hr-HPV were detected in 90.3% and 76.8%, respectively. HPV16 was detected in 53.6% of HPV-positive LSIL. Of 19 hr-HPV-negative-LSIL, 86.7% (13/15 tested) were lr-HPV-positive. Of all LSIL, 39.0% demonstrated a 'viral' Ki-67 (Figure 4A–D). p53 mid-epithelial staining was seen in 25.6% of LSIL, both in p16^{INK4a}-positive and -negative lesions (Figure 4E–H).

HPV-INDEPENDENT VIN

Of 46 HPV-independent VIN, 39 (84.8%) had originally been reported as HSIL. Mutant p53 staining was present in 65.2%: 41.3% with positive staining and 23.9% with null staining (Figure 5A–D). The remainder 34.8% had wildtype, scattered p53 staining (Figure 5E–H). p53 mid-epithelial staining was not observed. In five HPV-independent VIN, HPV was detected (four hr-HPV and one lr-HPV), all in combination with mutant p53 and negative $p16^{INK4a}$. Morphology was heterogeneuos, with nondifferentiated ('HPV-associated-like') morphology in 41.3% (Figure 6A–H). Of those, 89.5% had mutant p53 staining, in 94.7% in combination with negative $p16^{INK4a}$ staining, and in 90.9% (10/11) of tested cases without hr-HPV. One HPV-independent VIN with nondifferentiated ('HPV-associated-like') morphology showed mutant p53 staining in combination with block-positive $p16^{INK4a}$ and negative HPV (Figure 6E–H).

NONDYSPLASTIC LESIONS

Nondysplastic, nonviral lesions exclusively showed negative $p16^{INK4a}$, negative HPV, and scattered wildtype p53 staining. Ki-67 was increased in ≤1/3 of the epithelium in 40.0% and in ≤2/3 of the epithelium in 5.7%. Nondysplastic lesions included LS (17.1%), inflammation (31.4%), reactive changes (31.4%), (fibro-)epithelial polyps (5.7%), and no abnormalities (14.3%).

VULVAR CANCER RISK IN PATIENTS WITH HG-VIN

Four patients (0.5%) with microinvasive disease at histopathological reassessment were excluded from the VSCC analyses. In HPV-associated HSIL, the 10-year cancer incidence was 8.0% (Table 4, Figure 7A). HSIL with vulvar carcinoma in follow-up tested for HPV (n = 59) harboured HPV16 in 86.4%, HPV18 in 3.4%, HPV18/hr-HPV non-16/18 undetermined ('variant X') in 1.7%, and HPV33 in 3.4%. In 6.8%, hr-HPV non-16/18 type was not further specified. The prevalence of HPV genotypes did not significantly differ between HSIL with or without VSCC in follow-up.

In HPV-independent VIN, the 10-year cancer incidence was 53.6%, 67.4% for HPV-independent VIN/p53mut and 27.8% for HPV-independent VIN/p53wt (P = 0.004). The 10-year cancer incidence in HPV-independent VIN with nondifferentiated ('HPV-associated-like') versus differentiated morphology was 73.7% versus 39.3% (P = 0.001), Figure 7B. Median time to cancer was significantly shorter for HPV-independent VIN compared to HSIL: 1.8 versus 6.0 years (P < 0.001), for p53 mutant versus wildtype IHC: 1.5 versus 5.1 years (P = 0.010), and for nondifferentiated ('HPV-associated-like') versus

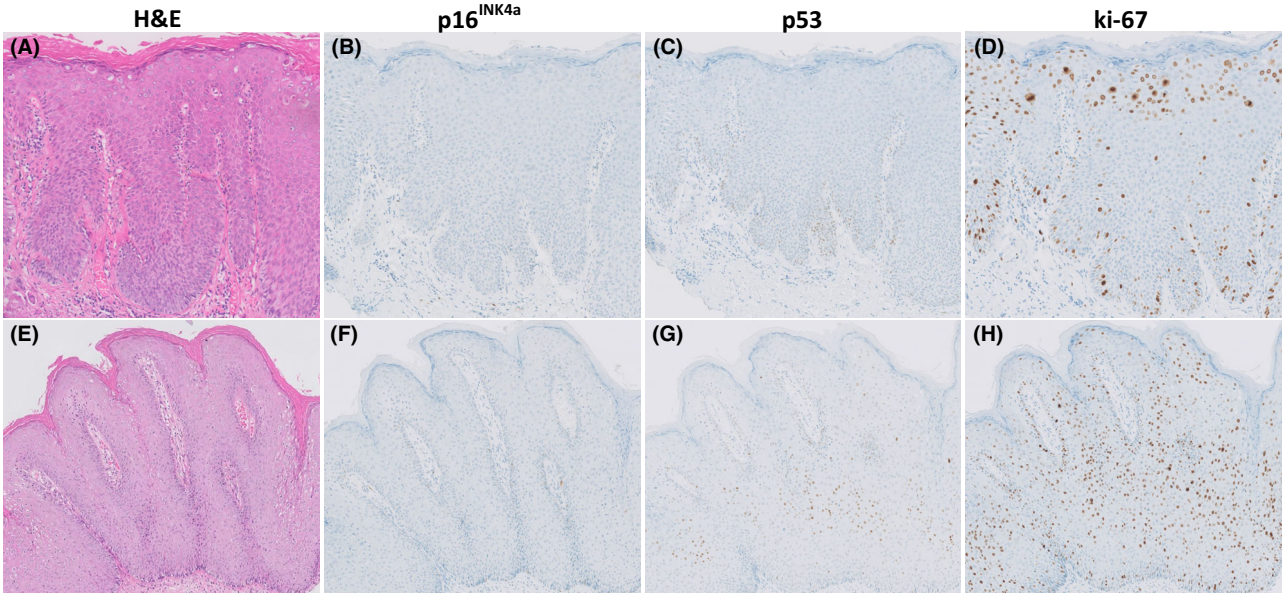

Figure 4. HPV-associated vulvar low-grade squamous intraepithelial lesion (LSIL). (A–D) LSIL with 'viral' Ki-67 in scattered individual koilocytic cells in the upper epithelium with lesser staining in the lower epithelium, mimicking transepithelial increased Ki-67. (E–H) LSIL with wildtype p53 mid-epithelial staining with sparing of the basal cell layer and negative $p16^{INK4a}$.

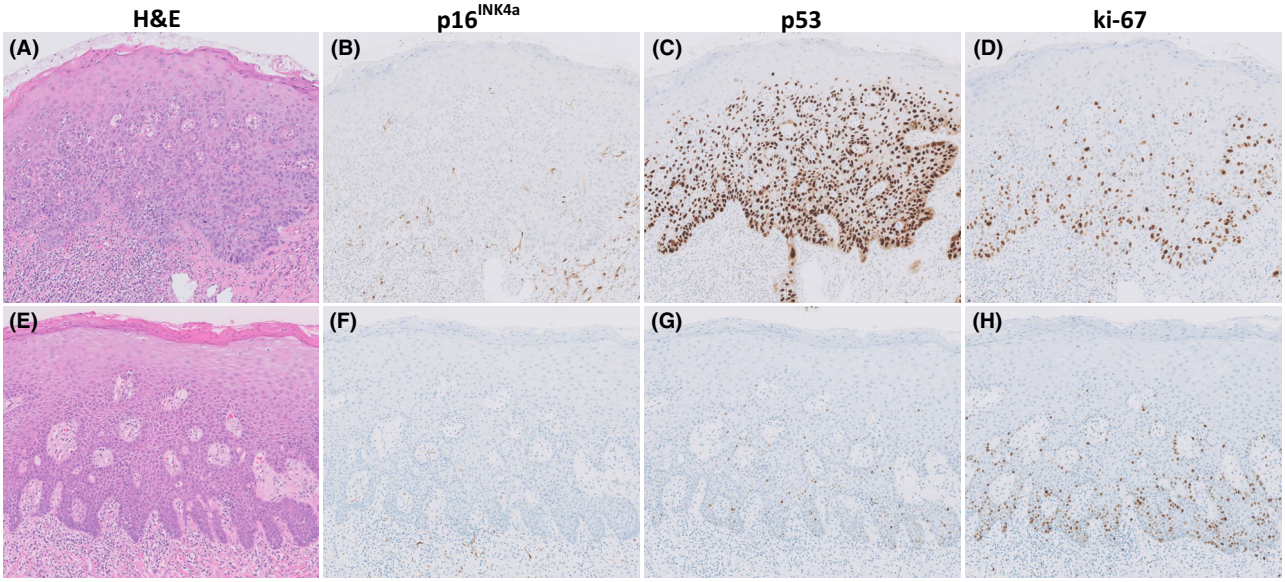

Figure 5. HPV-independent vulvar intraepithelial neoplasia (VIN). (A–H) Representative examples of HPV-independent VIN with differentiated morphology, negative p16^{INK4a} and respectively mutant (C) versus wildtype scattered p53 staining (G).

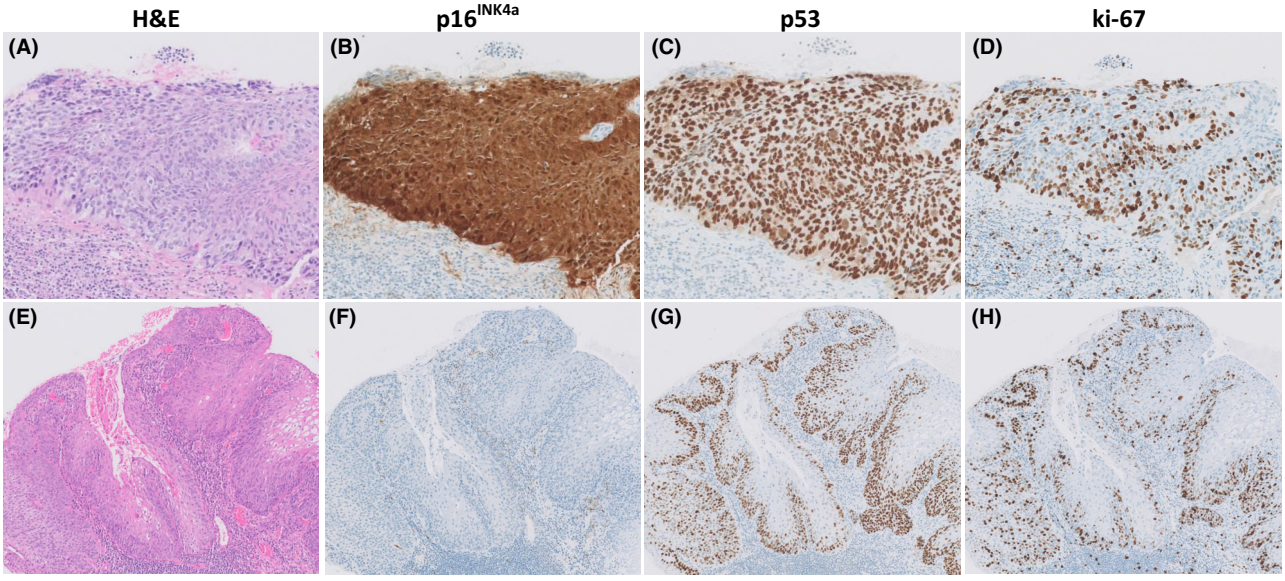

Figure 6. HPV-independent vulvar intraepithelial neoplasia (VIN) with nondifferentiated ('HPV-associated-like') morphology. (A–H) Both cases had negative HPV DNA and mutant positive p53 staining. In (A–D), basaloid morphology is seen, whereas in (E–H) wide and deep rete ridges, with moderate pleomorphism and koilocytic-like changes are seen.

differentiated morphology: 0.5 versus 3.2 years (P = 0.002).

Discussion

Our study on vulvar lesions of 751 patients, all originally reported as hg-VIN, demonstrated that immunohistochemical markers p16^{INK4a} and p53 are very valuable for adequate categorization into HPV-associated and HPV-independent types. Given the broad morphologic spectrum of HPV-independent VIN, including the morphological overlap with HPV-associated SIL, the original categorization based on H&E staining without the use of IHC led to an

Table 4. Risk of vulvar squamous cell carcinoma (VSCC), including time to VSCC, per disease category

	Absolute VSCC risk	Cumulative incidence of VSCC (95% CI)			Median time to VSCC,
	No. (%)	1 year	5 years	10 years	years (range)
HPV-associated HSIL	61/578 (10.6)	2.1 (0.9–3.3)	4.5 (2.7–6.3)	8.0 (5.8–10.2)	6.0 (0.3–24.2)
HPV-independent VIN	25/46 (54.3)	19.6 (8.2–31.0)	45.7 (31.4–60.0)	53.6 (38.7–68.5)	1.8 (0.3–10.9)
HPV-ind VIN/p53 mutant	20/30 (66.7)	30.0 (13.5–46.5)	63.3 (46.1–80.5)	67.4 (50.3–84.5)	1.5 (0.3–6.7)
HPV-ind VIN/p53 wildtype	5/16 (31.3)	0.0 (NA)	12.5 (0.0–28.8)	27.8 (3.9–51.7)	5.1 (1.2–10.9)
HPV-ind VIN/ differentiated	11/27 (40.7)	3.7 (0.0–10.8)	25.9 (9.4–42.4)	39.3 (19.9–58.7)	3.2 (0.8–23.3)
HPV-ind VIN/ nondifferentiated	14/19 (73.7)	42.1 (20.0–64.2)	73.7 (53.9–93.5)	73.7 (53.9–93.5)	0.5 (0.3–16.5)

HSIL, High-grade squamous intraepithelial lesion; HPV-independent VIN, Human papillomavirus-independent vulvar intraepithelial neoplasia; NA, Not applicable.

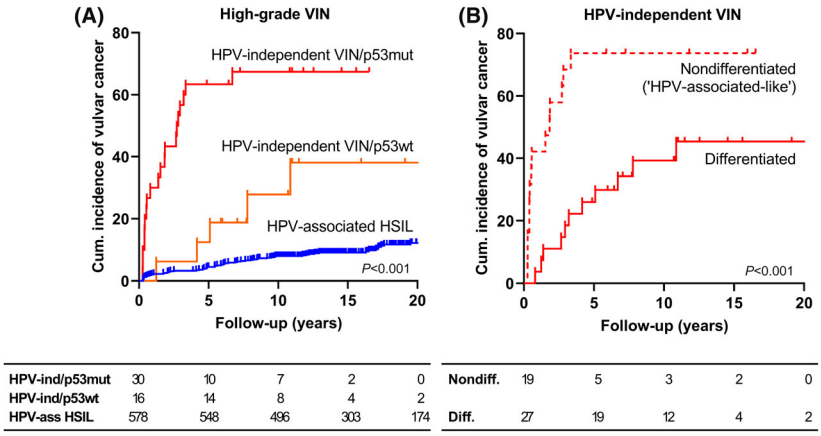

Figure 7. Cumulative incidence of vulvar cancer in high-grade VIN. (A) Stratified for three subtypes of high-grade VIN: HPV-associated HSIL, HPV-independent VIN/p53 mutant, and HPV-independent VIN/p53 wildtype. (B) HPV-independent VIN with differentiated morphology versus nondifferentiated ('HPV-associated-like') morphology. HPV-independent VIN, Human papillomavirus-independent vulvar intraepithelial neoplasia; HSIL, High-grade squamous intraepithelial lesion.

inadequate diagnosis of 84.8% of HPV-independent VIN. Typing of VIN is of utmost importance, given the different 10-year cancer risk of 8.0% for HPV-associated HSIL and 53.4% for HPV-independent VIN. Strikingly, HPV-independent VIN/p53mut had a twice as high 10-year cancer risk compared to HPV-independent VIN/p53wt (67.4% versus 27.8%). In addition, HPV-independent VIN with nondifferentiated ('HPV-associated-like') morphology had the highest 10-year cancer risk of 73.7%, compared to a 39.3% risk with differentiated morphology ($P < 0.001$). Besides the higher cancer risk, both p53 mutant and nondifferentiated subgroups of HPV-independent VIN had a much shorter time to cancer progression.

HPV-ASSOCIATED VULVAR LESIONS

Consistent with the literature, 99.0% of HSIL were $p16^{INK4a}$ block-positive and 98.9% harboured hr-HPV, mostly HPV16.[22] Among HSIL progressing to vulvar cancer, 86.4% had HPV16, which was not statistically different from HSIL without progression, possibly because the vast majority of HSIL were HPV16-positive.

In LSIL, we observed p16^{INK4a} in 30.5% and hr-HPV positivity in 76.8%, which is both higher compared to the literature, reporting rates of respectively 4–20% and 10–42%.[23–26] A likely explanation is that the LSIL in our study comprised a selected series, all originally diagnosed as hg-VIN. Characteristic of LSIL in our series was the high proportion (39.0%) of 'viral' Ki-67 staining, with increased numbers of positive cells in the upper epithelial layers compared to the lower epithelial layers. A 'viral' Ki-67 staining pattern was not observed in HPV-independent VIN, and therefore it can aid in distinguishing it from LSIL, as HPV-independent VIN and LSIL may show morphologically overlapping features with negative p16^{INK4a} and wildtype p53. To our best knowledge, 'viral' Ki-67 staining has only been described once before, in 2/11 HPV-positive vulvar seborrheic keratoses.[27] It is important to recognize a 'viral' Ki-67 pattern, as it may erroneously lead to upgrading and overtreatment of vulvar lesions.

Both wildtype p53 staining with reduced intensity and mid-epithelial patterns were also exclusively seen in HPV-associated SIL, and should not be confused with true mutant patterns, as seen in HPV-independent VIN.[9,17,28,29] While *Tp53* mutations can occur in HPV-associated SIL, they are usually nonfunctional.[30,31] These cases show combined p16^{INK4a}-positive/p53 wildtype patterns, indicating that hr-HPV drives the pathogenesis in these lesions. Reduced p53 in HPV-associated SIL is likely explained by p53 degradation by the E6 protein of oncogenic HPV.[32,33] p53 mid-epithelial staining is not fully understood and has been described for vulvar, anal, and cervical precursors.[31,34,35] One likely explanation is that an E6 splice variant is expressed that cannot degrade p53.[36,37]

HPV-INDEPENDENT VULVAR LESIONS

The diagnosis of HPV-independent VIN is challenging, as shown by multiple reports in recent years.[8,13,38–40] The histomorphology of HPV-independent VIN displays a broad spectrum, from characteristic HPV-independent VIN to more subtle changes of precancerous tissue. Molecular aberrations can extend beyond the epithelium with only deceptively minimal cytologic atypia, as recently described.[38,41]

The frequency of mutant p53 staining in HPV-independent VIN in our series was 65.2%, which is in line with other series, describing rates from 42% to 100%.[11,42] Interestingly, HPV-independent VIN/p53mut had a significantly higher cancer risk compared to HPV-independent VIN/p53wt. In addition, 41.3% of HPV-independent VIN in our series had nondifferentiated ('HPV-associated-like') morphology, a lesion type first described in 2009 and histologically indistinguishable from HPV-associated SIL.[14] This subset of HPV-independent VIN had the highest 10-year cancer risk (73.7%) and the shortest median time to carcinoma (0.5 years). Possible explanations are the high rate of mutant p53 IHC, or the basaloid histology, which is associated with worse prognosis in other carcinoma types, especially in SCC of the head and neck.[43] Our results are consistent with the recent recognition that VSCCs with mutant p53 or nondifferentiated ('HPV-associated-like') morphology exhibit higher recurrence rates and poorer survival than their counterparts.[39,42,44] All these observations highlight the importance of using the biomarkers p16^{INK4a} and p53 IHC for VIN typing.

In addition to dVIN, the 2020 WHO classification of female genital tumours has included two HPV-independent VIN/p53wt lesions: differentiated exophytic vulvar intraepithelial lesion (DEVIL) and vulvar acanthosis with altered differentiation (VAAD).[1] DEVIL is defined by an exophytic growth pattern and the absence of significant nuclear atypia.[45,46] In our study, most HPV-independent VIN/p53wt were not exophytic, a few showed marked atypia, and some showed nondifferentiated morphology. Therefore, the term 'HPV-independent VIN/p53wt' probably better delineates the disease than the former terms VAAD, DEVIL, VAM (vulvar aberrant maturation), and vaVIN (HPV-independent VIN/p53wt verruciform acanthotic VIN).[47] It should be emphasized that VAAD was not encountered in our series because those lesions have originally not been reported as hg-VIN. HPV-independent VIN/p53wt precursors likely have a broader morphological spectrum than currently described. Verrucous lichen simplex chronicus carries a relatively high cancer risk, but is often still regarded as reactive instead of a premalignant lesion. Given the morphologic overlap with reactive lesions, objective biomarkers are needed to identify HPV-independent VIN/p53wt vulvar lesions with a high cancer risk. DNA methylation has shown promising results, with an 87% detection rate in HPV-independent VIN.[48,49] Alternatively, CK17 and SOX2 immunohistochemistry showed a higher expression in HPV-independent VIN compared to nondysplastic vulvar tissues, but more studies are needed.[50,51]

HPV DNA testing is useful in some cases, but one should be aware of the pitfalls. Mere detection of HPV DNA or positive p16^{INK4a} alone does not prove a functional role of HPV. In our series, one HPV-independent VIN had mutant positive p53 and positive p16^{INK4a} IHC, and was classified as HPV-independent VIN, given the negative HPV DNA. Positive p16^{INK4a} in this case

was not caused by hr-HPV, but possibly by a mutation in *CDKN2A*.[30] Hr-HPV was detected in 10.9% of HPV-independent VIN, all with mutant p53 staining and negative $p16^{INK4a}$. Two other studies have shown comparable high numbers, of 6.4% and 12.5%.[42,52] A possible explanation for the high HPV prevalence in HPV-independent VIN is the presence of LS, in which defective viral clearance or reactivation of a latent HPV infection can occur, because of prolonged use of topical corticosteroids.[53]

We acknowledge several limitations of our study. Given the retrospective nature of this study, clinical information was limited and was not used for categorization of the lesions. Besides adaptations in international classification systems, both the use of IHC and the awareness of HPV-independent VIN increased during the study period (1991–2011), limiting direct comparison of the initial pathology report to current practice.[1,54] Also, we have not been able to confirm p53 IHC with p53 mutational status, although the concordance is known to be high (91–97%).[16,17,55]

Our study also has several strengths. To the best of our knowledge, this is the largest study that has comprehensively characterized vulvar lesions, originally diagnosed as hg-VIN with respect to IHC of $p16^{INK4a}$, p53, and Ki-67, including HPV genotyping and long-term vulvar cancer risk. Selection of our cohort was population-based instead of institutional-based. Correlations between morphology, HPV genotype, and vulvar cancer risk have not been established before in hg-VIN. We used a standardized and clinically validated methodology to detect HPV DNA, allowing our results to provide valuable data on the expected effect of vaccination in The Netherlands.

Conclusion

We were the first to demonstrate in a large population-based series that HPV-independent VIN with p53 mutant IHC or nondifferentiated ('HPV-associated-like') morphology has distinctive pathological and behavioural features. Both subtypes are highly aggressive and warrant closer surveillance after surgery. In order to allow correct typing of hg-VIN, the performance of $p16^{INK4a}$ and p53 IHC on at least each newly diagnosed VIN lesion is highly recommended. Future work should focus on clinicopathological and molecular factors searching for additional biomarkers, which are necessary for accurate diagnosis of HPV-independent VIN and for cancer risk stratification of HPV-associated SIL.

Author contributions

Nikki Thuijs: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Validation; Visualization; Writing –original draft; Writing – review & editing. Marc van Beurden: Methodology; Funding acquisition; Supervision; Visualization; Writing – review & editing. Sylvia Duin: Investigation; Resources; Writing – review & editing. Daniëlle Heideman: Formal analysis; Validation; Writing – review & editing. Johannes Berkhof: Formal analysis; Funding acquisition; Methodology; Supervision; Validation; Visualization; Writing – review & editing. Renske Steenbergen: Data curation; Funding acquisition; Methodology; Supervision; Validation; Visualization; Writing – review & editing. Maaike Bleeker: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Supervision; Validation; Visualization; Writing – review & editing.

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Conflict of interest

D.A.M.H. and R.D.M.S. are minority shareholders of Self-screen B.V., a spin-off company of VUmc. Self-screen B.V. holds patents related to this work, and develops, manufactures, and licences the high-risk HPV assay and methylation marker assays for cervical cancer. N.B.T., S.D., M.v.B., J.B., and M.C.G.B. declare no conflicts of interest.

Ethics approval and patient consent

This study was approved by the local Medical Ethics Committee of Amsterdam UMC, location VUmc (reference number 2017.561). Informed consent was not required.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Details of tissue processing, immunohistochemistry of p16INK4a, p53 and Ki-67, DNA isolation and HPV DNA testing.

Table S1. Table overview of reaching final classification of 741 originally diagnosed high-grade VIN.