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A panel of p16^{INK4A}, MIB1 and p53 proteins can distinguish between the 2 pathways leading to vulvar squamous cell carcinoma

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Two pathways leading to vulvar squamous cell carcinoma (SCC) exist. The expression of proliferation- and cell-cycle-related biomarkers and the presence of high-risk (hr) HPV might be helpful to distinguish the premalignancies in both pathways. Seventy-five differentiated vulvar intra-epithelial neoplasia (VIN)-lesions with adjacent SCC and 45 usual VIN-lesions (32 solitary and 13 with adjacent SCC) were selected, and tested for hr-HPV DNA, using a broad-spectrum HPV detection/genotyping assay (SPF$_{10}$-LiPA), and the immunohistochemical expression of MIB1, p16^{INK4A} and p53. All differentiated VIN-lesions were hr-HPV- and p16-negative and in 96% MIB1-expression was confined to the parabasal layers. Eighty-four percent exhibited high p53 labeling indices, sometimes with parabasal extension. Eighty percent of all usual VIN-lesions were hr-HPV-positive, p16-positive, MIB1-positive and p53-negative. Five (of seven) HPV-negative usual VIN lesions, had an expression pattern like the other HPV-positive usual VIN lesions. In conclusion, both pathways leading to vulvar SCC have their own immunohistochemical profile, which can be used to distinguish the 2 types of VIN, but cannot explain differences in malignant potential.

Key words: vulvar carcinoma; vulvar intraepithelial neoplasia; p16^{INK4A}; p53; MIB1; HPV

Vulvar squamous cell carcinoma (SCC) accounts for 3–4% of all female genital cancers. There are 2 types of vulvar SCC that have different clinical and pathological features.[1,2] Both types of vulvar cancer are preceded by their own type of vulvar intra-epithelial neoplasia (VIN). On the basis of histopathological characteristics, VIN lesions can be divided into usual VIN (also known as Bowenoid or classic VIN, basaloid or warty subtype) and differentiated VIN (formerly named simplex VIN or well-differentiated VIN).[3] Recently, the International Society for Vulvovaginal Disease (ISSVD) has proposed a revised nomenclature for vulvar lesions.[2,3]

The majority of vulvar SCCs occur in elderly patients with lichen sclerosus and develops following an human papillomavirus (HPV)-negative pathway.[4,5] Its premalignancy, differentiated VIN, can be difficult to distinguish from a benign vulvar lesion (*e.g.* chronic inflammation) or normal epithelium.[5,6] It is assumed that differentiated VIN is highly proliferative and might rapidly progress into an invasive neoplasm, because it is seldom found without (micro-invasive) vulvar carcinoma and often adjacent to HPV-negative vulvar SCC.[2,5,6] Since differentiated VIN is often unifocal and the amount of skin involved is limited, surgical treatment by means of a wide local excision probably reduces the risk of progression to invasive carcinoma.[7]

Usual VIN is often multifocal, occurs in younger women and is associated with smoking and HPV, predominantly HPV-16 and -18, and can lead to HPV-positive vulvar SCC.[8] One third of all vulvar SCCs is associated with HPV.[9] The risk of malignant transformation of usual VIN to an invasive carcinoma appears to be 3–4%. The viral gene products E6 and E7 interfere with 2 pathways of cell cycle regulation. HPV E6 can interact with p53, leading to p53 dysfunction, which allows for an absence of cell cycle arrest.[6,10] HPV E7 can inactivate pRb which can result in an overexpression of p16^{INK4A} and hyperproliferation.[11,12]

Proliferative activity in tissues can be visualized using MIB1, a proliferation marker which is a monoclonal antibody against the Ki-67 nuclear antigen, present in human proliferating cells in all stages of the cell cycle besides the G$_0$ phase.[13] In several (pre-) malignant lesions, MIB1-expression can be used for grading, estimating prognosis and prediction of biological behavior.[14–18]

The tumor suppressor p53 detects genetic alterations in cells in G$_1$-phase, resulting in cell cycle arrest or apoptosis. It is frequently mutated in HPV-negative vulvar SCC.[19] Immunohistochemically, p53 is detected frequently in vulvar SCC and differentiated VIN, most likely because of cellular accumulation of the mutated abnormal protein.[6]

The lack of knowledge about the oncogenesis of vulvar SCC and the malignant potential of VIN lesions result in the absence of an evidence based protocol for the optimal treatment and follow-up for patients with VIN. The aim of the present study was to investigate the patterns of MIB1, p16^{INK4A} and p53-expression and the presence of HPV in VIN lesions and adjacent SCCs to gain insight in the oncogenesis of vulvar SCC, and test whether these parameters can be helpful to distinguish the 2 types of VIN lesions.

Material and methods

Patients and histopathology

All patients with a histological diagnosis of VIN with or without concurrent primary vulvar carcinoma between 1990 and 2002, with available microscopic slides and paraffin blocks, were selected from the database of the Department of Pathology of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands (n = 162). No recurrent vulvar carcinomas were selected. When a patient had a VIN lesion preceding or after the diagnosis of vulvar carcinoma, only the carcinoma and the adjacent VIN lesion were used for analysis. This leads to a reduction with 25 cases. Another 17 patients were excluded because of the absence of VIN according to current criteria, in which VIN1 is no longer considered to be a premalignancy.[3]

All original hematoxylin-and-eosin-stained slides were reviewed by 1 pathologist with special expertise in gynecopathology [JB]. The histological diagnosis of the vulvar lesion was based on Kurman *et al*., Sideri *et al*. and Wilkinson *et al*.[3,20,21] The differentiation grade of vulvar SCCs was determined according to WHO criteria. In Figures 2*a*, 2*e*-inset, 3*a*, and 3*a*-inset, H&E

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stained sections of, respectively, solitary usual VIN, SCC associated with usual VIN and differentiated VIN with adjacent SCC are shown.

After revision, a total number of 120 patients with a VIN lesion were eligible for analyses. Eighty-eight patients had an associated primary vulvar carcinoma and 32 patients did not have nor developed a vulvar carcinoma (last date of follow-up December 2006). No patients with differentiated type VIN without a previous or subsequent vulvar SCC were diagnosed and therefore this entity was not present in this study. Of the 88 patients with an associated vulvar carcinoma, 13 had a concurrent usual VIN lesion and in 75 patients the carcinoma was adjacent to a differentiated VIN lesion. Representative sections for each case were selected for immunohistochemical analysis. A minimum distance of 0.5 cm between differentiated VIN and SCC in the same slide was required. When normal vulvar epithelium was available in the tissue sample, a site most distant from the (pre-) malignant vulvar lesion was selected for analysis of one or more immunohistochemical parameters ($n = 62$; 40 patients with a differentiated VIN lesion with associated SCC, 9 patients with usual VIN with associated SCC and 13 patients with a solitary usual VIN lesion).

Material of 32 patients was also used in previous studies by the same group; mostly providing lichen sclerosus and normal vulvar epithelium (not in investigation in this article).[9,22] When the use of VIN and/or SCC was duplicated, new H&E staining as well as immunohistochemical- and HPV-analysis was performed.

Recently, a patient with a solitary dVIN lesion was treated at our hospital. She had lichen sclerosus and 5 years ago she underwent a hemivulvectomy with bilateral inguinofemoral lymph node dissection because of a multifocal, macro-invasive SCC of the vulva. Afterwards she received radiotherapy because of 2 positive lymphnodes in the left groin.

HPV DNA detection

Four micrometer thick tissue sections of each archival sample were put into a reaction tube and incubated overnight at 56°C in 200 μl of 10 mM tris-HCL with 1 mM EDTA, 0.2% Tween-20, and proteinase K (0.3 mg/ml). If the VIN lesion and vulvar carcinoma were not available in the same archival tissue sample, 2 tissue sections (placed in 1 reaction tube) were used for HPV analysis. Proteinase K was inactivated by 10 min incubation at 100°C. The sample was centrifuged for 10 min at 11.000 rpm and 10 μl was directly used for PCR analysis. A water blank control was processed with each batch of 10 samples. Broad-spectrum HPV DNA amplification was performed using a short PCR fragment (SPF-PCR) assay. The SPF-PCR system amplifies a 65 bp fragment of the L1 open reading frame, allowing for the detection of at least 43 HPV genotypes. Subsequent HPV genotyping was performed via a reverse hybridization line probe assay (HPV SPF_{10} Line BLOT 25, LABO Bio-Medical products B.V., Rijswijk, The Netherlands), allowing for simultaneous typing of the following 25 HPV-genotypes: HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70 and 74. The combined SPF-PCR-LiPA system for detection and genotyping of HPV has been described in detail elsewhere and is considered highly sensitive.[23,24] In cervical cancer studies, the HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 have been classified as high-risk and the HPV types 26, 53 and 66 as probable high-risk types of HPV.[12,25] The HPV types detected in this study were classified accordingly.

Immunohistochemistry

Serial tissue sections (4-μm thick) of formalin-fixed and paraffin-embedded blocks were cut with the first and the last sections hematoxylin and eosin-stained for control. After deparaffinizing and hydration, endogene peroxidase was blocked by incubation in 1.5% H_2O_2 in phosphate-buffered saline (PBS) for 15 min. Antigen retrieval was performed by microwave heat induction. The slides were preincubated with 20% normal goat serum (10 min) and then incubated with primary antibodies p53 (clone DO7, Dakocytomation, Denmark) 1:400, $p16^{INK4A}$ (clone 16PO4, Neomarkers, Fermont, CA) 1:500, and MIB1 (clone MIB1, Dakocytomation, Denmark) 1:200, all suspended in 1% bovine serum albumine (BSA)/PBS (60 min, RT). Subsequently, postantibody blocking was done for 15 min (powervision plus). This was followed by incubation with polymeric-horse-radish peroxidase-goat anti-mouse/rabbit/rat IgG (30 min, RT). The slides were developed with diaminobenzidine (mixed with H_2O_2) and the p53 and $p16^{INK4A}$ slides were rinsed in $CuSO_4$ for amplification; all slides were counterstained with Mayer's hematoxylin (30 sec), dehydrated and finally mounted. All incubation steps were followed by 3 washes in PBS. Titration experiments were performed to determine the aforementioned optimal dilutions for the primary antibodies and in each series a positive control was included (CIN3 lesion).

Quantification of immunohistochemical results

The immunoreactivity of $p16^{INK4A}$ in the VIN lesion (and, when present, in their adjacent vulvar carcinoma and normal tissue) was scored based on the localization and extent of the $p16^{INK4A}$-immunoreactivity within the epithelium. Three categories were discerned: (*i*) no $p16^{INK4A}$-positivity, (*ii*) focal $p16^{INK4A}$-positivity and (*iii*) diffuse, transepidermal positive $p16^{INK4A}$-staining.[26–28] For statistical purposes, focal $p16^{INK4A}$-staining was considered '*negative*.'

For MIB1, the localization of the immunoreactivity within the epithelium was assessed, and four categories were discerned: (*i*) basal or parabasal staining, (*ii*) positivity confined to cells in the lower one third of the epithelium, (*iii*) staining of cells in the lower two thirds of the epithelium, or (*iv*) diffuse, transepithelial positive staining.[9] For statistical purposes, MIB1-staining in the (para)-basal layers or in the lower one-third of the epithelium was considered 'low' and MIB1-staining in the lower two-thirds or the entire epithelium was considered 'high.'

For p53, cells were considered to be positive in case of nuclear staining. The extent of p53-positivity was evaluated by determining the percentage of p53-positivity in basal layer cells after counting 200 consecutive cells (labeling index (LI)). The pattern of p53-staining was assessed by recording the location of the positive cells in the levels of the epithelium. The term "suprabasal extension" was used when p53-positive cells were found in both the basal layer and in higher layers of the epithelium.[5] In carcinomas, the percentage of p53-positive cells were estimated after evaluation of the entire lesion present in the slide.

When the carcinoma was micro-invasive ($n = 6$, 4 adjacent to usual VIN, 2 adjacent to differentiated VIN) no immunohistochemical staining results could be scored.

Statistics

On the basis of the histological diagnosis, patients were divided in 3 groups (usual VIN with SCC, usual VIN without SCC and differentiated VIN with SCC). The difference in age was tested using the nonparametric Kruskal–Wallis test. Differences in presence of HPV, $p16^{INK4A}$-expression and MIB1-localization were tested using the χ^2 test. The differences in p53-LI in VIN lesions and p53-positivity in SCCs between groups were tested using the nonparametric Mann-Whiney-*U* test. For all analyses a p-value of <0.05 was considered to be statistically significant.

Results

Patients with a vulvar carcinoma adjacent to usual type VIN had a lower median age (52 years, SD 13.4 years) compared to patients with differentiated VIN with associated carcinoma (74 years, SD 12.5 years). Patients with usual VIN without an associated vulvar carcinoma had a median age of 36 years (SD 10.8 years) at the time of diagnosis. The differences in age at the time of diagnosis were highly statistically significant (Kruskal-Wallis

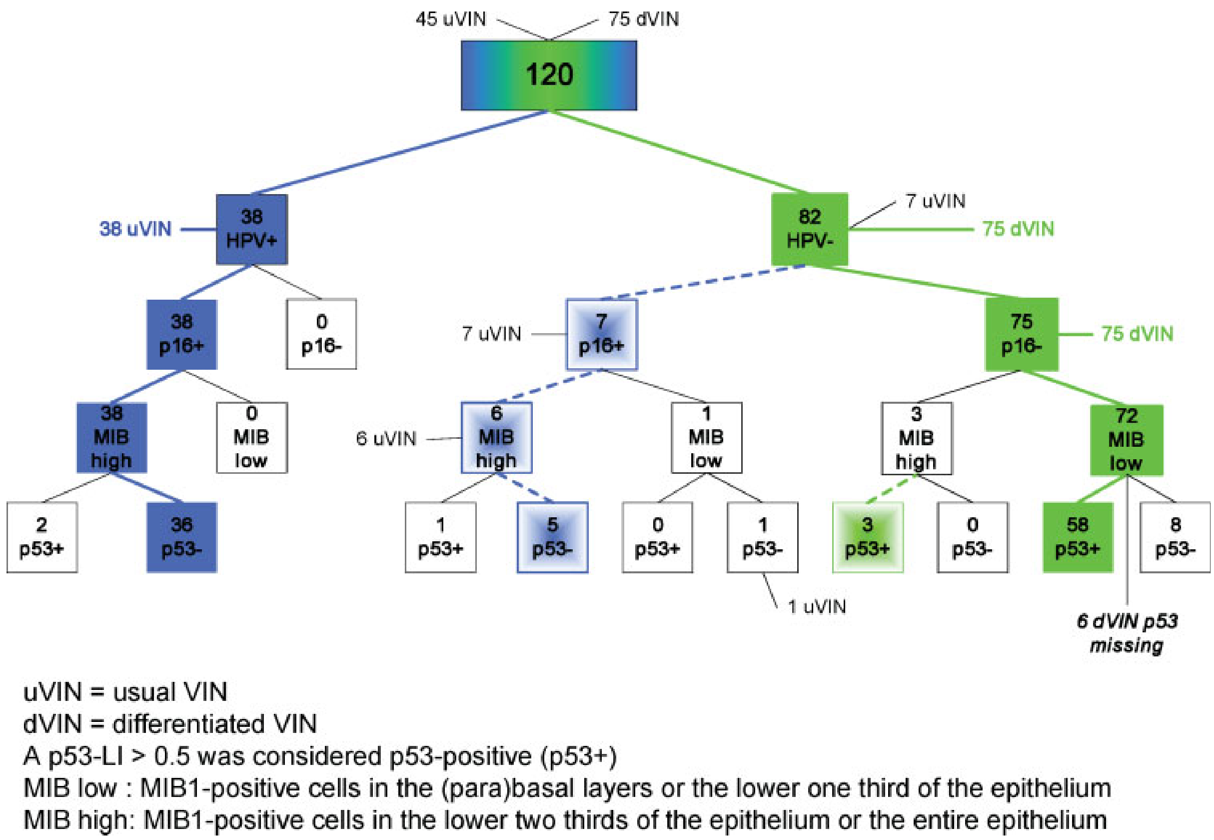

Figure 1 – Flow diagram showing simultaneous HPV-positivity, p16^{INK4A}-expression and p53-LI in the 120 lesions. A p53-LI > 0.5 was considered p53-positive (p53+). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Test, $p < 0.001$). In sixty percent of the patients with differentiated VIN a (clinical and/or histological) diagnosis of LS was noted in the patient chart; in this study no examination for LS on the histology specimen was performed.

The FIGO and TNM stages of the vulvar carcinomas in both groups were comparable: 64% of the dVIN associated carcinomas were FIGO stage I or II *vs*. 75% of the usual VIN related carcinomas. Ninety-three percent of the dVIN-associated carcinomas were T1/T2 and 63% were N0 *vs*. 83% and 75% of the usual VIN related carcinomas.

In Figure 1, the combined results of the presence of HPV and the expression of p16^{INK4A}, MIB1 and p53 are summarized in a flow-chart.

HPV

Usual VIN was significantly more often high-risk (hr)-HPV positive than differentiated VIN; 38 of 45 cases of usual VIN were hr-HPV positive (84%); all cases of differentiated VIN were hr-HPV-negative (χ^2, $p < 0.001$). Usual VIN without associated carcinoma showed comparable percentages of positivity for hr-HPV with usual VIN with associated carcinoma; 26 of 32 cases (81%) and 12 of 13 cases (92%) respectively (data not shown, χ^2, $p > 0.05$). One usual VIN lesion without associated SCC was positive for low-risk HPV (HPV 6). All hr-HPV-positive usual VIN lesions were diffusely positive for p16^{INK4A} and had MIB1-expression up to high in the epithelium. Ninety-five percent (36/38) had a p53 LI of ≤ 0.5.

p16^{INK4A}

The staining pattern of p16^{INK4A} in usual VIN was cytoplasmic and nuclear, with more nuclear than cytoplasmic staining (see Fig. 2*b*). Irrespective of the type of adjacent lesion, normal tissue showed either no or minimal immunostaining for p16^{INK4A}.

The p16^{INK4A} immunoreactivity in vulvar carcinomas showed similarities with the p16^{INK4A} immunoreactivity in its associated VIN lesion: all usual VIN lesions were positive for p16^{INK4A}, whereas in differentiated VIN, all 75 lesions were negative for p16^{INK4A}. All usual VIN lesions without SCC were positive for p16^{INK4A}. In the group of differentiated VIN lesions, only one case was positive for p16^{INK4A}. The difference in p16^{INK4A}-positivity in the carcinomas adjacent to differentiated VIN (3/73:4%) and usual VIN (8/9:89%) was significant (data not shown, χ^2, $p < 0.001$).

MIB1

A uniformly nuclear and mostly very strong MIB1-staining was seen in all types of vulvar lesions, with no cytoplasmic staining (see Figs. 2*c*, 2*e* and 3*b*). MIB1 immunoreactivity in normal epithelium (irrespective of the type of adjacent VIN lesion) was parabasal with a negative basal cell layer in all cases.

In SCC adjacent to differentiated VIN the median estimated positivity for MIB1 was 70% (range 10–100%) whereas in SCC adjacent to usual VIN the median estimated MIB1-positivity was 80% (range 50–100) (Mann-Whitney-*U*, $p = 0.06$). There was a significant difference in the localization of MIB1-staining between the 2 types of VIN lesions (χ^2, $p < 0.001$); usual VIN lesions showed MIB1 staining up to high in the epidermis in 44 of 45 of cases (98%), in contrast to the MIB1-staining confined to the lower layers of the epithelium in differentiated VIN in 72 of 75 cases (96%).

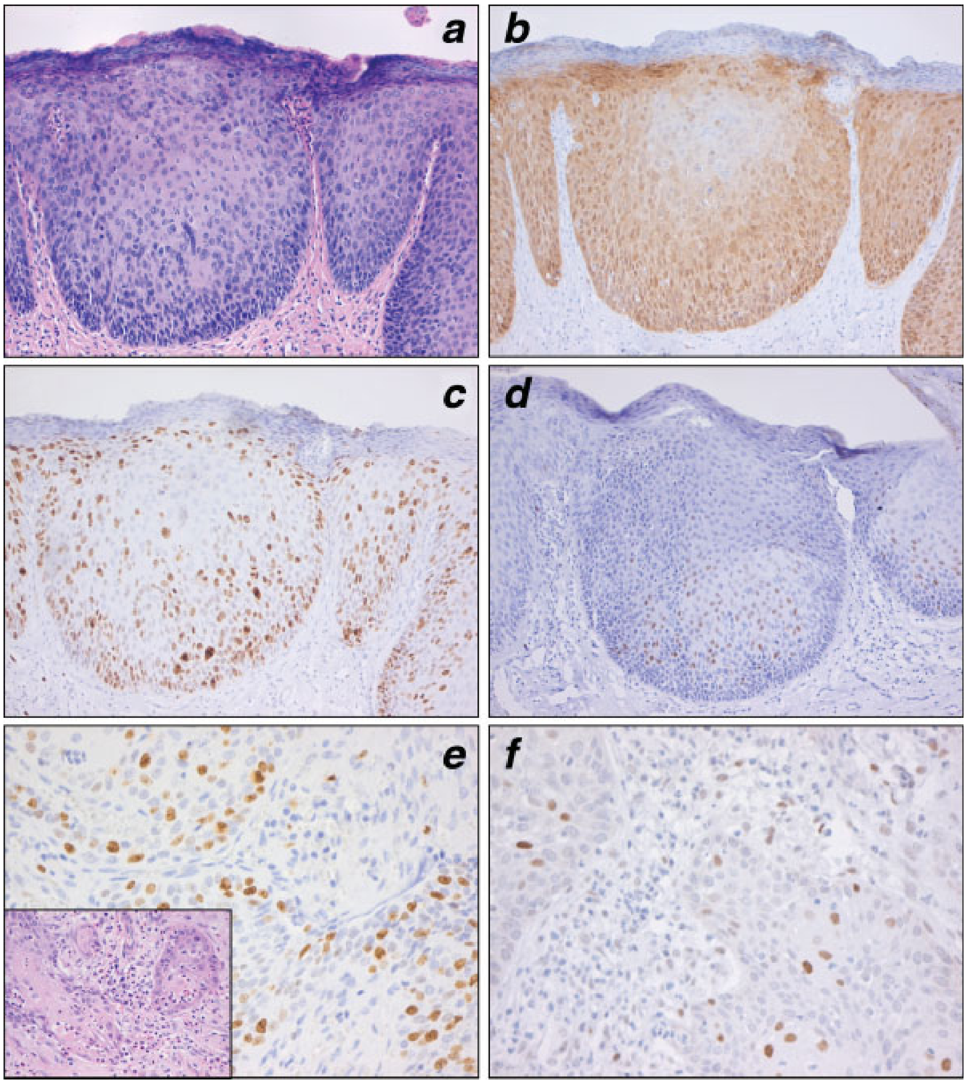

Figure 2 – Solitary usual VIN lesion (*a–d*) and squamous cell carcinoma adjacent to a usual VIN lesion (*e*,*f*). (*a*) H&E stained slide of a usual VIN lesion without adjacent squamous cell carcinoma; atypical nuclei can be found throughout the entire epithelium. (*b*) The entire epithelium is positive for p16^{INK4A}. (*c*) MIB1-positive cells can be found in at least the lower 2/3 of the epithelium in usual VIN. (*d*) Clusters of p53-positive cells can be found in the epithelium of a usual VIN lesion. (*e*) MIB1-postive nests in vulvar squamous cell carcinoma. On the H&E photo in the inlay, mitotic figures and atypia can be seen. (*f*) In the carcinoma adjacent to usual VIN, around 25% of the cells are positive for p53. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

p53

Analyzing the p53-expression patterns in VIN lesions, 2 distinct patterns became apparent. In differentiated VIN lesions, the basal cell layer often was positive for p53, and in most lesions there was "suprabasal extension" as can be seen in Figure 3*c*. In usual VIN lesions less cells of the basal layer were positive. The suprabasal positivity in usual VIN, occasionally showed a distinct clustered pattern, in which central parts of the epithelial rete ridges were positive for p53 whereas the rest of the epithelium was negative for p53 as can be seen in Figure 2*d*. In normal vulvar epithelium no p53-expression was found. The expression of p53 in the 2 types of carcinoma can be found in Figure 1*f* (carcinoma adjacent to usual VIN) and Figure 3*d* (carcinoma adjacent to differentiated VIN).

The median LIs and the percentages of p53-positivity in the carcinomas are shown in Table I. Analyzing the p53 LIs revealed that the p53 LI in differentiated VIN adjacent to VC was significantly higher than the p53 LI in usual VIN adjacent to VC (Mann-Whitney-*U*, $p < 0.001$). The difference in p53 LIs between usual VIN with and without VC was not significant (Mann-Whitney-*U*, $p < 0.08$). The median percentages of p53-positivity in vulvar carcinoma adjacent to differentiated VIN were significantly higher than in vulvar carcinoma adjacent to usual VIN (Mann-Whitney-*U*, $p = 0.008$).

Discussion

Two separate pathways lead to the development of vulvar SCC, which have their own precursor lesions, with a unique immunohistochemical profile that corresponds with the profile in the adjacent carcinoma. We believe that the use of this immunohistochemical profile can be of particular help in the correct and timely diagnosis of VIN.

In VIN lesions as well as the adjacent carcinomas, the expression of p16^{INK4A} was highly associated with the presence of HPV. This close relation has already been demonstrated in the vulva,[9,29,30] the cervix,[31] the head and neck region,[32,33] the skin[34] and the anorectal region.[35] In the oral cavity, immunohistochemi-

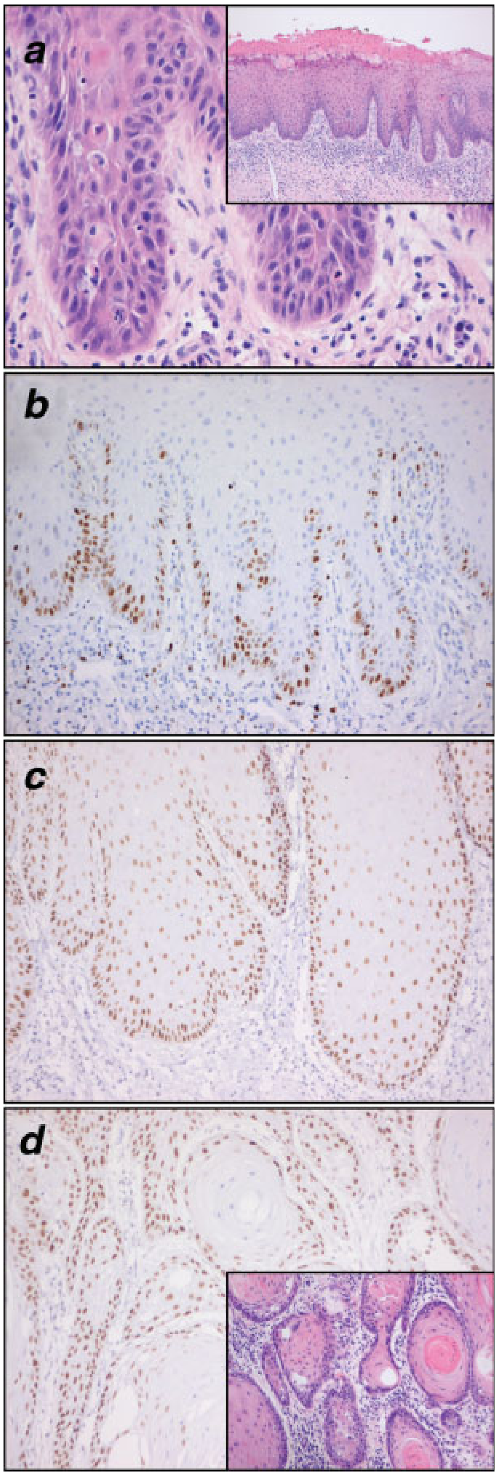

FIGURE 3 – Differentiated VIN lesion with adjacent squamous cell carcinoma. (*a*) H&E stained slides of a differentiated VIN lesion (adjacent to squamous cell carcinoma). Nuclear atypia and the presence of mitotic figures in the differentiated VIN lesion is confined to the basal cell layers. Hyperkeratosis and dyskeratosis are present and the rete ridges are elongated. (*b*) In differentiated VIN, MIB1-positivity is confined to the basal and parabasal layers of the epithelium. (*c*) In differentiated VIN, p53-posititivity is most prominent in the basal cell layers with suprabasal extension. (*d*): Around 90% of the cells of the invasive nests of the squamous cell carcinoma adjacent to differentiated VIN are positive for p53. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I – p53 LIs AND RANGE IN VULVAR CARCINOMA AND VIN

	Median	Range
Vulvar carcinoma (74[1])	%	<min–max>
Adjacent to differentiated VIN (66)	67.5%	<0–95>
Adjacent to high-grade VIN (8)	30.0%	<15–60>
VIN (114[2])	LI	
Differentiated type (69)	0.85	<0.16–1.00>
High-grade VIN, adjacent to VC (13)	0.025	<0.00–0.12>
High-grade VIN, without VC (32)	0.058	<0.00–0.55>

VC, vulvar carcinoma.
[1]When the carcinoma was micro-invasive (n = 6) p53 positivity could not be estimated and in 8 cases there was no carcinoma left in the p53 slide (but was present in H&E), therefore the total number of carcinomas in this table is less than 88.–[2]In 6 cases there was no VIN lesion left in the p53 slide (but was present in H&E), therefore the total number of VIN lesions is less than 120.

cal p16^{INK4A} detection has proven to be fully equivalent to HPV detection.[36] Others have shown that clinically meaningful viral HPV infections can be reliably measured with an algorithm of p16^{INK4A} immunostaining followed by PCR on p16^{INK4A}-positive cases.[33] In this study, the use of p16^{INK4A} alone was sufficient to identify all usual VIN lesions, and the immunohistochemical profiles of 5 of the 7 HPV-negative usual VIN lesions (see Fig. 1) suggest that even though we used a highly sensitive and accurate HPV detection method,[24] the results in at least 5 usual VIN lesions were false-negative.

High-HPV DNA was found in only 12/88 (14%) of all the vulvar SCCs in this study, all adjacent to a usual VIN lesion and HPV16 was, as in earlier publications on HPV in the genital area, most prevalent.[8,37] Previous studies on vulvar carcinomas have reported hr-HPV infection in 0–57% of the cases, depending on the HPV detection method and the types of SCC that were analyzed.[9,19,38–40]

Similar to the previously published series of Yang and Hart, the differentiated VIN-lesions adjacent to SCC showed a high p53 LI and a comparable expression-pattern with suprabasal extension.[5] Usual hr-HPV-positive VIN lesions, however showed a much lower p53 LI-positive VIN lesions and the lower percentage in the HPV-positive carcinomas in our study, which has also been described by others.[41–43] As previously described by Santos *et al*., in HPV-positive SCCs, p16^{INK4A} and p53 tended to be mutually exclusive.[30] Nogueira *et al*. described comparable results for VIN in women younger *vs.* older than 55 years of age, without testing for HPV.[44] It is likely that the group of women younger that 55 years consisted of mainly high grade, HPV-positive, usual VIN lesions whereas the group of women over 55 years probably mainly consisted of HPV-negative, differentiated VIN lesions. The clustered positivity in the epithelium of usual VIN has never been described.

In normal vulvar tissue, irrespective of the adjacent type of VIN, no expression of p53 and p16^{INK4A} was found and MIB1-expression in normal vulvar tissue was confined to the lower one-third of the epithelium. As was shown in a recent publication, MIB1 can be used to distinguish normal vulvar epithelium from differentiated VIN and other premalignancies because of a MIB1-negative basal cell layer in normal vulvar epithelium.[22] This feature combined with the absence of expression of the cell cycle related proteins investigated in this study, can improve the timely recognition of differentiated VIN as it is often overlooked or mistaken for a benign dermatose such as pseudoepitheliomatous hyperplasia and lichen simplex chronicus.[45,46] The differences in age of the 2 groups of VIN lesions were highly statistically significant. This fits the epidemiological data known from literature; usual VIN occurs in younger women and LS associated differentiated VIN and keratinizing vulvar SCC occurs at a higher age.

The fact that no isolated, solitary differentiated VIN lesions have been found in this study supports the idea that differentiated

VIN is a lesion with a short intra-epithelial phase that rapidly progresses to vulvar SCC.[6,7] This is supported by the fact that, in this study, all differentiated VIN lesions presented adjacent to vulvar SCC, and mostly had a size of more than 1 cm. We strongly believe in the high malignant potential of differentiated VIN. Incidental cases of differentiated VIN occurred in our hospital, but all after a patient had been treated for vulvar SCC. We also found some cases of differentiated VIN on biopsy, and invasive carcinoma in the subsequent vulvectomy or excision (performed within 2 weeks of diagnosis). Nevertheless, there is controversy regarding the actual role of differentiated VIN in the development of vulvar SCC. It has also been described as the *in situ* carcinoma component adjacent to the invasive carcinoma,[47] and our results cannot confirm nor reject this hypothesis. Furthermore, differentiated VIN can be difficult to diagnose, both clinically and histopathologically.[42,45,46,48] When differentiated VIN is found in the surgical margins of an excision, this might have consequences for the further treatment and follow-up of the patient. Better recognition and uniform use of nomenclature will facilitate future research and the comparison of published results. The changes in nomenclature of squamous vulvar lesions proposed by the ISSVD are not yet uniformly used.[3]

In conclusion, both pathways leading to vulvar SCC have their own molecular background. Future studies should focus on the exact role of p53 in the development of HPV-negative vulvar SCC and the malignant potential of differentiated VIN. Using a robust immunohistochemical panel with the proteins p16^{INK4A}, p53 and MIB1, the 2 types of VIN lesions can be accurately distinguished and recognized. Timely diagnosis and thus early recognition of differentiated VIN lesions should lead to a more extensive treatment strategy for this kind of VIN lesion.

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