Vulvar cancer : pathogenesis, molecular genetics and treatment

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CHAPTER 6

Stathmin is a highly sensitive and specific biomarker for vulvar high-grade squamous intraepithelial lesions

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Abstract

Aims: Differentiating between HPV-dependent vulvar low-grade and high-grade squamous intraepithelial lesions (LSILs and HSILs) remains difficult in selected cases. Stathmin, a protein involved in cell cycle progression, might be a useful additional marker for this differentiation. The aim of this study is to investigate the additional diagnostic value of stathmin expression in vulvar intraepithelial neoplastic (VIN) lesions.

Methods: Immunohistochemical analysis was used to evaluate stathmin, P16 and Ki67 expression in 91 samples, including LSILs (n=16), HSILs (n=50), differentiated VIN (dVIN; n=10), lichen sclerosis (LS; n=10), and normal vulvar tissue (n=5).

Results: Stathmin was expressed in more than one-third of the epithelium in all HSILs and in 20% of LSILs. P16 and Ki67 were expressed in more than one-third of the epithelium in 94% of HSILs and in 13% and 40% of LSILs, respectively. Stathmin was expressed in more than one-third of the epithelium in 10% of the dVIN and in none of the LS or normal lesions. P16 and Ki67 expression was not present in more than one-third of the epithelium in any of these lesions. The sensitivity of stathmin for differentiating between LSILs and HSILs was 100% compared to a sensitivity of 94% for both p16 and Ki67. The specificity of stathmin, p16 and Ki67 was 80%, 87% and 60%, respectively.

Conclusions: Stathmin is a highly sensitive and specific biomarker for the diagnosis of vulvar HSIL. In addition to the more commonly used immunohistochemical markers p16 and Ki67, stathmin can be a useful diagnostic tool for identifying HSILs, especially in cases in which differentiating between LSIL and HSIL is difficult.

Introduction

Treatment of vulvar precursor lesions is a challenge for gynaecologists, and accurate differentiation between high-grade and low-grade vulvar precursor lesions is important for their clinical management (1). The nomenclature for vulvar lesions has changed in the last years. The most recent classification system of WHO (2014) and the International Society for the Study of Vulvar Disease (ISSVD, 2015) endorses a two-tiered system for human papilloma virus (HPV)-dependent intraepithelial lesions as low-grade squamous intraepithelial lesions (LSILs; flat condyloma, formerly termed vulvar intraepithelial neoplasia (VIN) 1) or high-grade squamous intraepithelial lesions (HSILs, formerly termed VIN2/3). Furthermore, this classification system discriminates between these HPV-dependent precursor lesions and the HPV-independent precursor lesion differentiated VIN (dVIN), which is associated with lichen sclerosis (LS) (2-5).

Vulvar LSILs encompass a range of HPV-associated vulvar lesions that are not pre-cancerous and do not require treatment unless they are symptomatic. In 90% of the vulvar LSILs, the associated HPV types are HPV 6 and 11 (6-8). Treatment can consist of the application of immunomodulating cream, podophyllin, cryotherapy, laser therapy or surgery (8-10). Vulvar HSIL is associated with high-risk HPV types, namely types 16 and 18, and has a 9%–16% chance of progression to vulvar squamous cell carcinoma (VSCC), if left untreated. The HPV-independent precursor lesion dVIN is an uncommon vulvar lesion that has been recognised as a distinctive diagnosis since the mid-1980s. The subtle clinical and histological changes make recognition and diagnosis difficult, which might contribute to the low prevalence (5,11,12). Importantly, the malignant potential of untreated dVIN lesions is probably as high as 80% (9,12,13). Given the malignant potential of HSILs and dVIN, it is important to treat these patients adequately and to ensure close follow-up. In addition, vulvar HSILs are often multifocal and are sometimes associated with cervical and vaginal intraepithelial neoplasia (13). For these reasons, it is clinically important to have an accurate histopathological diagnosis and to reliably distinguish between LSILs, HSILs and dVIN (1). Tangential sectioning, small biopsies, thermal artefacts, coexistent inflammatory or reactive epithelial atypia (with or without LS) and the application of subjective criteria all contribute to the difficulty of VIN diagnosis and grading (10,13). Two studies have investigated interobserver variability between LSIL and HSIL vulvar lesions and found moderate-to-good agreement of 73.9% and 82%, respectively (1,14). Experienced gynaecological pathologists show good agreement (67%) in distinguishing HPV-dependent from HPV-independent vulvar lesions (15), but the histopathological diagnosis of dVIN is more difficult, and the interobserver and intraobserver variability is high (11).

Currently, immunohistochemical staining of p16, p53 and Ki67 is widely used for the differential diagnosis of vulvar precursor lesions. P16, a cyclin-dependent kinase-4 inhibitor, is especially useful for differentiating between HPV-dependent VIN (p16-positive) and dVIN (p16-negative). The E6 and E7 proteins of oncogenic HPV bind and inactivate p53 and pRb, leading to unregulated cell proliferation. This results in compensatory expression of the p16 tumour suppressor protein; thus, immunohistochemical staining of p16 is an accurate marker for HPV (10,11,13,16-18). However, p16 staining can be less specific for differentiating between vulvar LSIL and HSIL lesions, since these lesions sometimes show similar p16 expression patterns. Furthermore, the p16 staining pattern is sometimes difficult to interpret due to differential staining intensity and patterns that can also be found in inflammatory vulvar disorders (9,13,19-21). Ki67, a cell proliferation marker, is widely used to differentiate between cervical LSILs and HSILs (9,13,20,22). Several studies have shown that increased expression of Ki67 is associated with higher cervical SIL grade. In particular, the sensitivity of Ki67 in detecting cervical HSIL is high (93%–95%) (20,22-24). Because of the similarities between cervical intraepithelial neoplasia and VIN lesions, Ki67 has become a commonly used marker for VIN lesions as well, (7,14) and Ki67 staining is useful for differentiating between dVIN and normal vulvar epithelium (9,11,13,14,25). In dVIN, Ki67 positivity is usually confined to the basal layers of the epithelium, while in normal vulvar epithelium, Ki67 staining is completely negative (11,13,14,25). Notably, few studies have investigated Ki67 expression in vulvar SILs (7,13,20).

In contrast to HPV-dependent VSCC and vulvar HSIL, the tumour suppressor gene *TP53* is frequently mutated in HPV-negative VSCC and in its precursor lesion, dVIN. Immunohistochemical staining of p53 can thus be used as a marker for discriminating between HPV-independent and HPV-dependent precursors. A mutation in *TP53* can result in one of two patterns of aberrant expression on immunohistochemical staining that is, either strong diffuse p53 staining or a complete absence of staining (17,26). Despite the value of these widely-used markers, there are cases in which differentiation is difficult, and p16, Ki67 and p53 staining do not give a definite diagnosis.

Stathmin-1, which this study refers to as stathmin, is a ubiquitous microtubule-destabilising phosphoprotein in humans that is involved in cell cycle progression (27,28). Stathmin regulates microtubule dynamics and is required for all cellular processes that involve microtubule rearrangement, mainly mitosis. Accordingly, stathmin activity is critically important for cell division (29,30). Stathmin has been postulated to be an immunohistochemical marker for differentiating between low-grade and high-grade intra-epithelial diseases (28,29,31). One study showed that stathmin staining had greater specificity (93%) than p16 staining (44%) for detecting cervical HSILs. Stathmin staining distinguished HSILs from the majority of LSIL precursors (28).

Another study investigated stathmin as a marker of early neoplasia in the fallopian tube and found that stathmin could discriminate between normal fallopian tube epithelium, tubal intraepithelial carcinoma and invasive serous carcinoma (29).
In this study, we investigated stathmin expression in normal vulvar mucosa, vulvar LSILs and HSILs, dVIN and LS to determine whether stathmin can serve as an additional marker for the diagnosis of vulvar HSIL. In addition, we investigated whether stathmin could discriminate between HPV-dependent and HPV-independent precursor lesions.

Materials and methods

Cases

A total of 86 vulvar samples (resection, *n*=38; biopsy, *n*=48) were obtained from the surgical pathology archives of the Leiden University Medical Center after approval by the institutional review board. The samples included vulvar LSILs (originally reported as VIN 1 or condylomata lesions, but referred to as LSILs in this study; *n*=15); HSILs (originally reported as VIN 2/3 lesions, but referred to as HSILs in this study; *n*=51); dVIN (*n*=10); or LS (*n*=10). In addition, we analysed five normal vulvar epithelium samples from patients who underwent labia reduction surgery and who gave permission for the use of the material for research purposes. An overview of the classification of the patient samples is given in table 1. H&E stained slides were re-reviewed by a gynaecological pathologist (TB), and the diagnosis was confirmed in 81 (95%) cases. In four cases, the initial diagnosis was adjusted. One LSIL was reclassified as a HSIL, and three HSILs were reclassified as LSILs. The revised diagnoses were used in the final analysis. The classification of the vulvar lesions was performed according to the criteria described in the WHO and ISSVD classification systems (2,3).

Table 1: Sample characteristics

	Total samples N= 91
LSIL	15 (16,5%)
HSIL	51 (56%)
dVIN	10 (11%)
Lichen Sclerosis	10 (11%)
Normal vulvar epithelium	5 (5,5%)

LSIL: Low grade squamous intraepithelial lesion
HSIL: High grade squamous intraepithelial lesion
dVIN: Differentiated vulvar intraepithelial neoplasia

Immunohistochemistry

All samples were evaluated for stathmin, p16 and Ki67 expression by immunohistochemistry, and the HPV-independent dVIN and LS samples were also evaluated for p53 expression. The HPV-dependent samples were not stained for p53, because of the expected wildtype expression pattern in these lesions (17,32,33). Serial sections of 4-µm thickness were cut from formalin-fixed paraffin-embedded specimen blocks and dried overnight at 37°C. The tissue sections were deparaffinised, rehydrated and incubated in 0.3% hydrogen peroxidase (H_2O_2) solution for 20 min to block endogenous peroxidase activity. Antigen retrieval was carried out by microwave treatment in 0.01 M citrate buffer (pH 6.0) for 12 min. Slides were incubated overnight at room temperature with a polyclonal rabbit antibody to stathmin (Cell Signaling Technology, Danvers, MA, USA; 1:50 dilution; clone # 3352), a monoclonal mouse antibody to p16 (M Tm Laboratories, Westborough, MA, USA; 1:50 dilution; clone E6H4), a monoclonal mouse antibody to Ki67 (Dako, Denmark; 1:100 dilution; clone MIB-1) and a monoclonal mouse antibody to p53 (Thermo Scientific, 1:2000 dilution; clone DO-7) diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). After washing with PBS, tissue sections were incubated with PowerVision-Poly/HRP (Immunologic, The Netherlands) for 30 min. Immunoreactions were visualised using 0.5% 3.3'-diamino-benzidine-tetrahydrochloride and 0.02% H_2O_2 in Tris-HCl. The sections were then counterstained with haematoxylin. Because many samples contained both lesional and non-lesional areas, we considered the non-lesional areas as internal controls. Furthermore, immunohistochemical stainings were performed in series and the study sets included at least some positive and negative cases. Therefore, we did not add an external positive and negative control sample.

Evaluation of stathmin, p16, Ki67 and p53 expression

Two independent observers (LSN and TB) scored the immunohistochemical patterns, and consensus was reached by discussing cases with discordant initial scores. Stathmin staining was scored as 0 (all cells negative), 1+ (positive staining in less than one-third of the epithelial thickness), 2+ (positive staining in one-third to two-thirds of the epithelial thickness) or 3+ (positive staining in more than two-thirds of the epithelial thickness). Because there is not yet a validated cut-off value for interpretation of stathmin staining, first we evaluated the stathmin staining patterns in five normal and five dysplastic vulvar lesions. We found that stathmin expression was sometimes present in the basal layers of normal vulvar epithelium, but it was not present in more than one-third of the epithelial thickness. Therefore, similar to the interpretation of Ki67 expression, we decided to use stathmin expression in more than one-third of the epithelium as the cut-off value for increased expression (13,16,28). For the statistical analysis and determination of specificity and sensitivity, the staining results were subdivided into two groups: cytoplasmic or nuclear immunoreactivity in less than one-third of the epithelial

thickness (all samples that were scored 0 and 1+) or more than one-third of the epithelial thickness (all samples that were scored 2+ and 3+). Immunostaining with p16 was considered positive when there was diffuse staining of epithelial cells (nuclear and/or cytoplasmatic) in more than one-third of the epithelium (28,34). Immunostaining with Ki67 was scored as expression present in more than one-third of the epithelium or in less than one-third of the epithelium (24). Immunostaining of p53 was scored as wild type (patchy basal positivity) or as an aberrant staining pattern (either a strong diffuse expression pattern when >25% of the cells showed strong positive nuclear staining, or a complete absence of staining) (17,26).

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics V.20.0; chi-squared tests were used to differentiate between HSIL and LSIL. The sensitivity and specificity of stathmin, p16 and Ki67 staining were calculated for diagnosing HSIL. In addition, the positive predictive value (PPV) and negative predictive value (NPV) of all of the immunohistochemical markers was determined.

Results

The immunohistochemical staining results for stathmin, p16 and Ki67 are summarised in table 2. Figure 1 shows examples of the staining results of samples categorised as LSIL, HSIL, dVIN and LS. The expression of stathmin was evaluated in the epithelial layers as well as in the stromal component. In the stromal component, we observed some positive staining in the immune infiltrate. Stathmin expression was completely absent in four (80%) of the normal vulvar epithelium samples. In one (20%) normal vulva sample, stathmin expression was present in less than one-third of the epithelium.

Table 2: Immunohistochemical results; the number of samples that were scored as expression in > 1/3th of the epithelium for stathmin, p16 and Ki67

Diagnosis	Stathmin	p16	Ki67
LSIL	3/15 (20%)	2/15 (13,3%)	6/15 (40%)
HSIL	51/51 (100%)	48/51 (94,1%)	48/51 (94,1%)
dVIN	1/10 (10%)	0/10 (0%)	0/10 (0%)
Lichen Sclerosis	0/10 (0%)	0/10 (0%)	0/10 (0%)
Normal vulvar epithelium	0/5 (0%)	0/5 (0%)	0/5 (0%)

LSIL: Low grade squamous intraepithelial lesion
HSIL: High grade squamous intraepithelial lesion
dVIN: Differentiated vulvar intraepithelial neoplasia

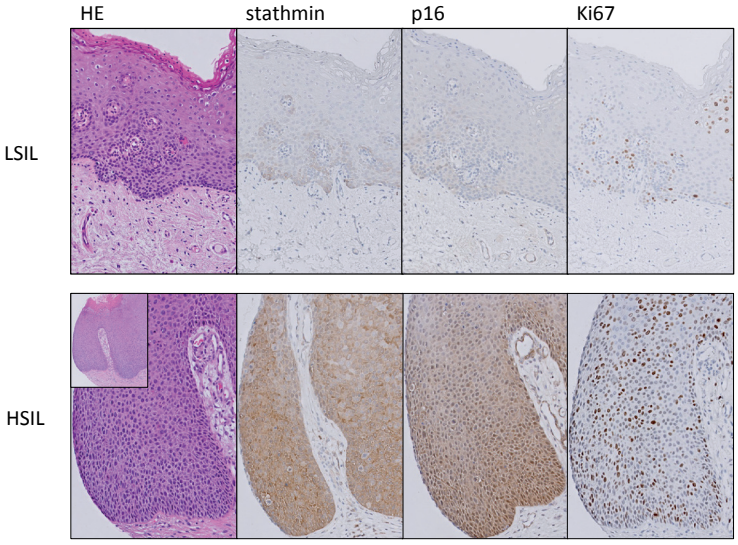

Figure 1a: H&E, stathmin, p16 and Ki67 staining patterns in a vulvar LSIL and HSIL. Inserted figure shows the HE of the HSIL on a lower magnification

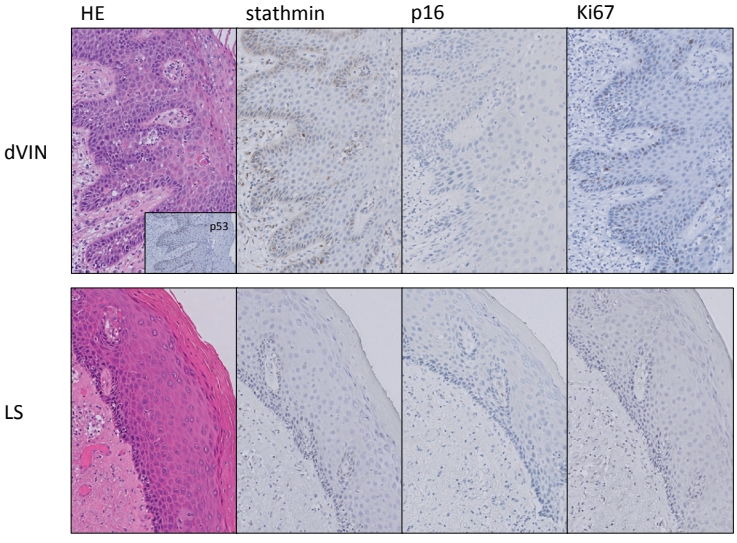

Figure 1b: H&E, stathmin, p16 and Ki67 staining patterns in a differentiated VIN (dVIN) and lichen sclerosus (LS) lesion. p53 staining in the dVIN sample showed a strong diffuse expression pattern, suggestive for a mutation in TP53 (inserted figure)

All of the HSILs showed stathmin expression in more than one-third of the epithelium. In 12 (80%) of the 15 LSILs, stathmin expression was confined to the basal layer of the epithelium (scored as 1+). The other three (20%) LSILs showed stathmin expression in more than one-third of the epithelium. Stathmin expression was completely absent (scored as 0) in four (40%) of the dVIN and seven (70%) of the LS lesions, and five (50%) of the dVIN and three (30%) of the LS lesions expressed stathmin in less than one-third of the epithelium. One (10%) dVIN sample showed stathmin expression in more than one-third of the epithelium. Staining with p16 was positive in more than one-third of the epithelium in 48 (94%) HSILs, and 2 (13%) of the LSILs also showed positive p16 staining in more than one-third of the epithelium. As expected, and in line with the initial diagnosis, all dVIN were completely negative for p16 expression (scored as 0). Seven (70%) of the 10 LS samples were completely negative for p16 staining, while the remaining 3 (30%) showed positivity in the basal keratinocytes (less than one-third of the epithelium). All normal vulvar epithelium samples were completely negative for p16 staining. Ki67 staining was present in more than one-third of the epithelium in 48 (94%) of the HSILs and in 6 (40%) of the LSILs. All other HSILs and LSILs showed Ki67 expression in the basal layer of the epithelium. All dVIN, LS and normal vulvar epithelium samples were scored as showing Ki67 expression in less than one-third of the epithelium. Of these samples, two (20%) of the dVIN and eight (80%) of the LS were completely negative for Ki67 staining.

Table 3 gives an overview of the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of stathmin, p16 and Ki67 for detecting vulvar HSILs. Stathmin showed higher sensitivity (100%) than p16 (94%) and Ki67 (94%). The specificity was lower than the sensitivity for all immunohistochemical markers: 80% for stathmin, and 87% and 60% for p16 and Ki67, respectively. The PPV was comparable for all markers, while the NPV was especially high for stathmin (100%) compared with p16 and Ki67 (NPVs of 81% and 75%, respectively).

Table 3: Sensitivity, specificity, positive predictive value and negative predictive value for differentiation between low- and high-grade vulvar squamous intraepithelial lesions

	Stathmin	p16	Ki67
Sensitivity (%)	100%	94%	94%
Specificity (%)	80%	87%	60%
PPV (%)	94%	96%	89%
NPV (%)	100%	81%	75%

PPV: positive predictive value
NPV: negative predictive value

We also evaluated the association between stathmin expression and p53 scoring in the HPV-independent dVIN and LS samples (Table 4). Seven (70%) of the dVIN samples were independent lesions and three (30%) adjacent to invasive cancer. Six of the seven independent dVIN lesions progressed towards invasive carcinoma during follow-up. In the dVIN cases, two (20%) were scored as having wild-type p53 expression (both were independent dVIN lesions, one with and one without progression towards VSCC), and the other samples (80%) showed an aberrant p53 staining pattern (either a strong diffuse expression pattern or no expression). In the LS cases, eight (80%) were scored having as wild type p53 expression, while the other two cases were scored as having aberrant p53 staining. All of the samples with p53 wild-type staining (*n*=10) showed stathmin expression in less than one-third of the epithelium. One of the dVIN cases with an aberrant p53 staining pattern showed stathmin expression in more than one-third of the epithelium.

Table 4: Association between stathmin expression and p53 staining in 20 HPV-independent vulvar precursor lesions (10 dVIN and 10 LS)

Stathmin	p53 wild type staining pattern	p53 aberrant staining pattern*
Expression in < 1/3th of the epithelium	10/10	9/10
Expression in > 1/3th of the epithelium	0/10	1/10

*Either highly expressed or completely absent P53 immunohistochemical staining indicating a possible P53 mutation

HPV: human papilloma virus
dVIN: differentiated vulvar intraepithelial neoplasia
LS: lichen sclerosis

Discussion

The aim of this study was to determine whether stathmin expression as measured by semiquantitative immunohistochemistry could improve the diagnosis and correct grading of vulvar SILs. Our findings indicate that stathmin is a highly sensitive and specific biomarker for the differentiation of vulvar LSILs and HSILs. The excellent sensitivity of stathmin (100%) exceeded that of the commonly used markers p16 and Ki67 (94% sensitivity for both). The specificity of stathmin was similar to that of p16 and exceeded that of Ki67.

In the most recent classification of vulvar squamous intraepithelial lesions the former VIN1 or flat condyloma has been adjusted towards LSIL, while the former VIN 2 and 3 or usual type VIN has been adjusted towards HSIL. In the new classification system,

dVIN remains a distinct entity (3,5). It is clinically important to differentiate between vulvar LSIL and HSIL, especially because of the malignant potential of vulvar HSIL, which is 9%–16% for untreated patients and 3% for patients who receive treatment. Spontaneous regression occurs in less than 1.5% of patients with vulvar HSIL patients, and it mostly occurs during the first 10 months following the diagnosis (35). In contrast, LSIL has a negligible chance of progression towards invasive VSCC (9,12,13). In view of these risks, it is clear that an adequate treatment plan is needed, especially for vulvar HSIL; current therapies include local treatment with the immunomodulating agent imiquimod, laser excision or surgery (9,12,13).
The commonly used immunohistochemical marker p16 is not always sufficient to differentiate between vulvar LSIL and HSIL (9,13). Additional use of Ki67 as a marker can help in this differentiation (14,25), but in some cases, doubt remains about the definite diagnosis. Our data show that stathmin expression in HPV-dependent vulvar dysplasia may be informative in cases in which there is doubt about the grading because of the high specificity and sensitivity of stathmin expression in more than one-third of the epithelium. After revision by an expert gynaecology pathologist, one case was upgraded from an LSIL to a HSIL, and in three cases, the initial diagnosis was revised from an HSIL to a LSIL. Interestingly, the Ki67 staining pattern was especially difficult to interpret in these three cases. Specifically, Ki67 staining was present in the parabasal layer, but it was also present in the upper epithelium, where koilocytic atypia is present, and this can easily be mistaken for an HSIL vulvar lesion. This Ki67 staining pattern has been described previously, but it is not well-known (6,7). Stathmin expression was negative in these samples, clearly demonstrating that stathmin staining is truly different from Ki67 staining.

Currently, there is no consensus on how to interpret the immunohistochemical staining results of stathmin expression. One study that looked at stathmin expression in cervical SIL defined increased stathmin expression as positive staining in more than two-thirds of the cervical epithelium (28). In contrast to that study, we used a cut-off of stathmin expression in more than one-third of the epithelium based on our preliminary evaluation of stathmin expression in normal vulvar epithelium and dysplastic lesions and based on our specificity and sensitivity results. When we used more than two-thirds of the epithelium as a cut-off value, the sensitivity decreased to 76% and the specificity increased slightly to 81%.

Morphological diagnosis of especially dVIN is difficult and interobserver variability is high (11). dVIN and LS are frequently associated with mutation in the *TP53* gene (12). Therefore, staining with p53 can be helpful to differentiate between dVIN or LS and vulvar SILs, although p53 is not necessarily a marker for dVIN (36). One of our samples diagnosed as dVIN showed a p53 wild-type expression pattern and did not progress

towards an invasive tumour. Consequently, it can be possible that this sample is not a genuine dVIN lesion. Previous studies have shown that there is a relationship between mutant p53 expression and increased stathmin expression in precancerous lesions (serous tubal intra-epithelial carcinomas (STICs)) of the fallopian tube (29,31). In these STICs, a *TP53* mutation results in upregulation of stathmin expression. The majority of *TP53* mutations result in loss of function. However, mutations in the *TP53* gene can also lead to a novel protein with a gain-of-function. Hypothetically, stathmin upregulation is needed to support this gain-of-function mutant p53 (29,31,37). Therefore, we evaluated the association of the p53 expression pattern with the stathmin expression pattern in HPV-independent vulvar precursors. Intriguingly, and in contrast with the association reported in STICs, we observed no relationship between immunohistochemical p53 and stathmin staining in HPV-independent vulvar precursors. This can be interpreted as arguing against a direct mechanistic link between *TP53* mutation and stathmin expression; at the very least, it shows that these two markers are unrelated in dVIN. Additional mechanistic studies are needed to gain a better understanding of this observation.

We are the first to describe stathmin staining in vulvar samples and this comes with some limitations. Although vulvar precursor lesions are uncommon, this was a relatively small study. Another limitation is the absence of a predetermined and validated cut-off value for increased stathmin expression. The cut-off value used in this study must be validated in an independent set of vulvar samples. Furthermore, we focused on the expression of stathmin in vulvar precursor lesions and therefore did not include VSCCs. Due to this focused approach, we are not informed about the expression and potential diagnostic utility of stathmin expression in vulvar carcinomas.

In conclusion, stathmin is a highly sensitive and specific biomarker for high-grade dysplasia of HPV-associated vulvar precursors. It can be used in addition to p16 and Ki67 staining in formalin-fixed, paraffin-embedded tissue sections using routine immunohistochemical procedures when differentiation between vulvar LSILs and HSILs is difficult. Before implementing stathmin in daily practice, validation in an independent cohort is necessary.

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