

# Phosphorylated S6 as an immunohistochemical biomarker of vulvar intraepithelial neoplasia

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**As life expectancy lengthens, cases of non-viral-associated vulvar squamous cell carcinoma and its precursor lesion, so-called differentiated vulvar intraepithelial neoplasia (VIN), continue to increase in frequency. Differentiated VIN often is difficult to recognize and failure to detect it before invasion results in morbidity and mortality. Thus, identification of a reliable biomarker for this type of lesion would be of great clinical benefit. Our recent studies have identified activation (ser235/236 phosphorylation) of ribosomal protein S6 (p-S6) in basal epithelial cells as an event that precedes and accompanies laminin  $\gamma^2$  overexpression in most preinvasive oral dysplasias. To test this as a potential biomarker of vulvar dysplasia, we immunostained seven differentiated VINs and nine papillomavirus-related 'classic' VINs, most of which were associated with carcinoma, for p-S6. All carcinomas, all differentiated VINs, and most classic VINs contained regions of p-S6 staining in the basal layer, whereas basal and parabasal cells of normal vulvar epithelium and hyperplastic and inflamed lesions lacking cellular atypia were p-S6 negative. Laminin  $\gamma^2$  was expressed in a subset of VINs, always occurring within basal p-S6 positive regions, as we had found previously for oral dysplasias. Lichen sclerosis is considered a potential precursor of vulvar carcinoma. Two lichen sclerosis lesions of patients with a concurrent carcinoma and one of six lichen sclerosis lesions without atypia or known concurrent carcinoma were basal p-S6 positive. In summary, there is a distinct difference in p-S6 basal cell layer staining between benign and neoplastic vulvar squamous epithelium, with consistent staining of differentiated VIN and of some lichen sclerosis lesions. These results support further studies to assess the potential of p-S6 as a biomarker to identify vulvar lesions at risk of progressing to invasive cancer.**

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Vulvar squamous cell carcinoma accounts for 5% of female reproductive and genital tract cancers in the United States, with 4500 women initially diagnosed and 1000 dying of this cancer in 2012.<sup>1</sup> There are two widely recognized oncogenic pathways to vulvar carcinoma.<sup>2</sup> The first, which occurs in 40% of cases, is initiated by infection by the high-risk human papillomavirus HPV16 and leads to the development of the so-called warty and basaloid types of vulvar carcinoma.<sup>3,4</sup> HPV16-initiated carcinogenesis involves genomic integration of the viral oncogenes encoding E6 and E7, which inactivate and degrade

the tumor suppressor genes pRb and p53, respectively. As a consequence, the cell cycle is deregulated, p16<sup>INK4A</sup> expression is induced but has no growth inhibitory effect on the pRb-inactive cells, and hyperplasia and dysplasia ensue.<sup>5</sup> HPV16-associated carcinomas are preceded by dysplastic lesions termed 'classic' vulvar intraepithelial neoplasia (VIN). Classic VINs tend to be multifocal, to arise in premenopausal women, and, with acquisition of further genetic changes, may progress to invasive carcinoma.<sup>6</sup>

The second pathway leading to vulvar carcinoma results from genetic and epigenetic changes in the absence of papillomavirus infection. Genetic alterations in the precursor lesions, so-called 'differentiated' VIN, include allelic imbalances, microsatellite instability, and p53 mutations.<sup>7–9</sup> Differentiated VINs tend to be unifocal, to affect older women, and to precede the majority (60%) of vulvar carcinomas.<sup>3,10</sup> Histopathologic changes in

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differentiated VINs can be very subtle and there are no reliable biomarkers to identify them with certainty, making their detection and diagnosis challenging. Vulvar carcinoma may also arise within nondysplastic or minimally dysplastic lichen sclerosus lesions, so may go unheralded until invasive carcinoma has developed.<sup>11–14</sup>

The incidence of VIN has increased >4-fold since the early 1970s.<sup>15</sup> Whereas classic VIN and its associated carcinoma are expected to decrease substantially in the future as the result of vaccination against high-risk papillomaviruses,<sup>16</sup> the incidence of differentiated VIN and its associated carcinoma is rising, likely owing to the increasing lifespan of our population. Immunohistochemical analysis for abnormal patterns of p16<sup>INK4A</sup>, p53, and MIB1/Ki-67 expression, separately or as a panel, can distinguish classic from differentiated VIN but these markers often fail to identify the latter.<sup>17–20</sup> We therefore sought to find a more reliable biomarker for differentiated VIN.

Abnormal, overexpression of the  $\gamma^2$  subunit of the stratified epithelial basement membrane protein Laminin-332 was identified long ago as an immunohistochemically detectable characteristic of many types of invasive carcinoma.<sup>21–23</sup> More recently it was found that laminin  $\gamma^2$  overexpression begins at the preinvasive dysplastic stage of neoplastic progression in the oral epithelium and interfollicular epidermis.<sup>24,25</sup> Squamous cell carcinomas of the interfollicular epidermis, oral cavity, oropharynx, and lung very frequently have gene mutations and amplifications that hyperactivate their EGFR/ERK signaling pathway.<sup>26–28</sup> Our recent study identified EGFR/ERK signal pathway hyperactivation as necessary and sufficient to drive laminin  $\gamma^2$  overexpression in cultured human premalignant epithelial and squamous cell carcinoma cell lines and also found that the HPV16 E6 oncoprotein also induces laminin  $\gamma^2$  overexpression.<sup>29</sup> Laminin  $\gamma^2$  overexpression proved to require the ERK-activated RSK kinase and to correlate with increased phosphorylation of the translation factor eIF4B at S442 and of ribosomal protein S6 at S235/236.<sup>29</sup> Increased S6(S235/236) phosphorylation (p-S6) proved to be detectable immunohistochemically in the basal cell layer of oral dysplasias and squamous cell carcinomas, preceding and accompanying laminin  $\gamma^2$  overexpression.<sup>29</sup> We therefore sought in the present study to determine whether p-S6 in the basal cell layer is a typical feature of VIN and other potentially premalignant vulvar lesions.

## Materials and methods

### Cases Examined

Sixteen cases of vulvar dysplasia were selected from the files of the Brigham and Women's Hospital Department of Pathology. The formalin-fixed and paraffin-embedded selected specimens included

seven differentiated VINs, six of which were associated with invasive carcinoma, and nine classic VINs, six of which were associated with invasive carcinoma. VINs were classified as classic or differentiated independently by two of the authors who are gynecologic pathology specialists (A.P.P. and C.P.C.), using conventional histopathological criteria<sup>30</sup> and their classifications concurred in all cases. For comparison, 13 vulvar lesions judged by morphologic criteria not to be dysplastic, 12 of which were not associated with VIN or carcinoma at the time of biopsy, were selected from Pathology Department files. These 13 lesions were evaluated and classified independently by one of us (C.P.C.) and by Brigham and Women's Hospital staff pathologists who examined the specimens to write the original clinical pathology reports. Their diagnoses concurred in 12 of the cases, with the exception being a lichen sclerosus lesion that was scored as non-dysplastic by C.P.C. and mildly dysplastic in the original pathology report.

### Antibodies

Antibodies used for immunostaining were: p-S6 (S235/236) (rabbit monoclonal antibody clone 91B2; Cell Signaling Technology, Danvers, MA, USA) at 1:100 dilution and laminin  $\gamma^2$  (mouse monoclonal antibody clone D4B5; Millipore, Billerica, MA, USA) at 1:100 dilution.

### Immunohistochemical Staining

Four-micrometer sections were stained with hematoxylin and eosin (H&E) and immunostained for p-S6 and laminin  $\gamma^2$ . In order to improve retention of antigens for immunostaining,<sup>24</sup> sections were not 'baked' at 50 °C after cutting as is the customary practice in pathology labs. Instead, they were warmed at 37 °C for 30 min before deparaffinizing in xylene and rehydrating through 100, 95, and 70% ethanol and PBS. Antigen retrieval was carried out in 0.1 mM citrate buffer (pH 6.0) by autoclaving for 15 min at 120 °C under pressure of 23 p.s.i. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 10 min. After a 20 min blocking step in 10% horse serum (Invitrogen, Carlsbad, CA, USA), sections were incubated with primary antibodies either for 1 h at room temperature or overnight at 4 °C (these methods proved to yield the same immunostaining patterns), followed by incubation with biotinylated secondary antibody (1:100) for 1 h and avidin/biotin/peroxidase complex (ABC; Vectastatin Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) for 45 min, with three PBS rinses between each step. Bound antibody was visualized with 3,3'-diaminobenzidine as a chromogen (EnVisionSystem, DAKO, Glostrup, Denmark).



Some of the specimens were counterstained with Harris hematoxylin (Fisher Scientific, Kalamazoo, MI, USA). Slides were coverslip-mounted with Permount (Fisher Scientific) and examined with a NIKON Eclipse TE2000-S microscope (Nikon, Melville, NY, USA) fitted with an Insight QE SPOT camera (SPOT Imaging Solutions, Sterling Heights, MI, USA). Images were captured using SPOT software.

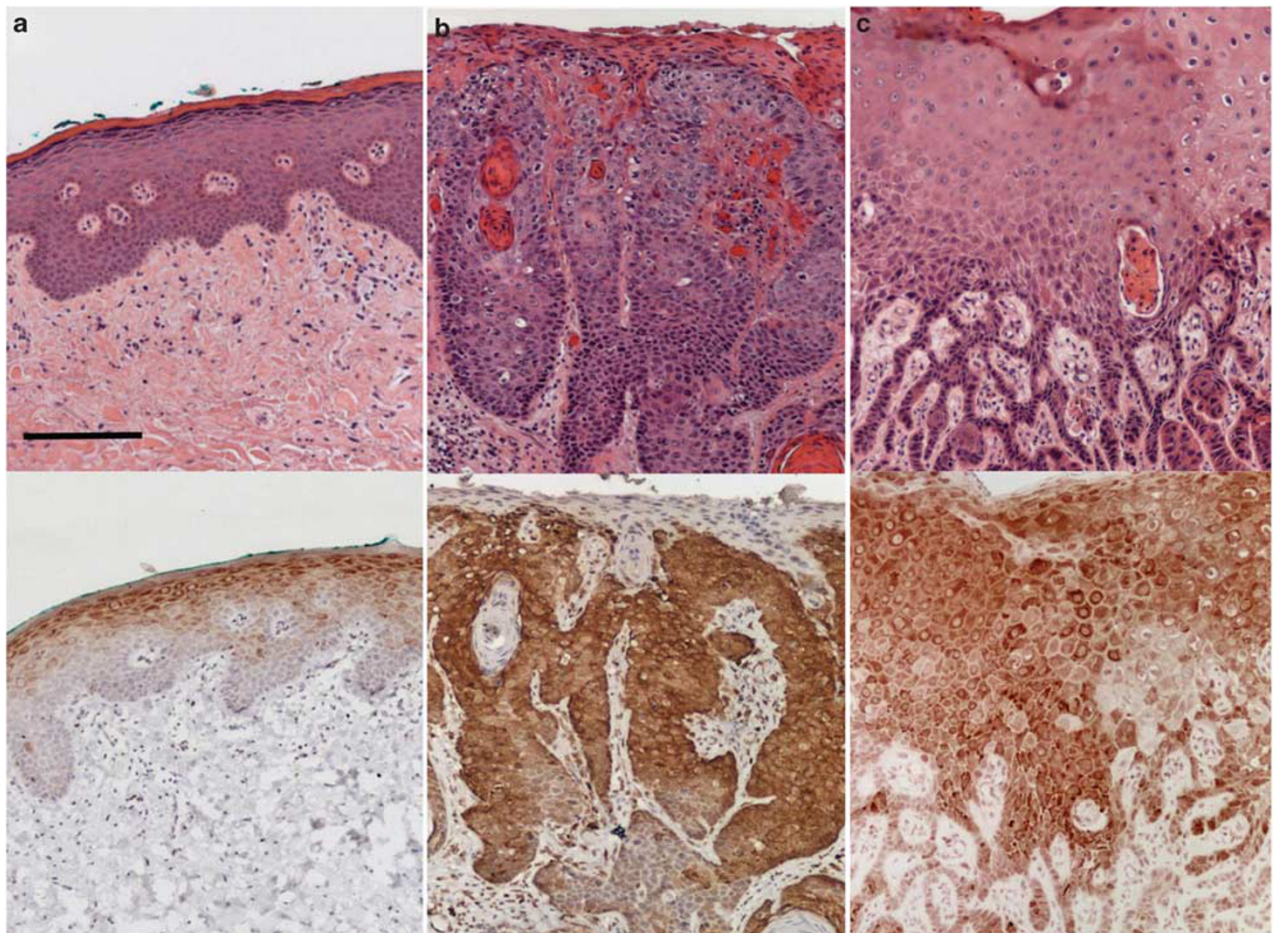
Some of the sections analyzed also contained regions of histologically normal epithelium, which served as a negative control to assess background staining. Immunostaining results were evaluated and photographed by two of the authors (A.P.P. and J.G.R.). P-S6 is present in the upper layers and absent in the basal and parabasal layers of normal oral epithelium, but frequently is present in cells of the basal layer in oral dysplasias and squamous cell carcinomas.<sup>29</sup> Laminin  $\gamma^2$  is undetectable in normal basal cells but frequently is present in the cytoplasm of basal cells in oral and epidermal dysplasias.<sup>24,29</sup> In the present study, p-S6 and laminin  $\gamma^2$  were

scored as positive when present in any basal cells in the section, regardless of the number or proportion of positive cells.

## Results

### P-S6 Immunostaining in Normal Vulvar Epithelium, Vulvar Carcinoma, and Classic and Differentiated VIN

Our recent identification of p-S6(S235/236) as a read-out for EGFR/ERK signal pathway hyperactivation in neoplastic oral epithelium and the presence of p-S6-positive basal cells in oral dysplastic lesions and squamous cell carcinomas<sup>29</sup> provided the rationale to examine VINs and vulvar carcinomas for this biomarker. We immunostained seven differentiated VINs, six of which were associated with carcinoma, and nine classic VINs, six of which were associated with carcinoma, for p-S6. Some of the specimens also contained histologically normal epithelium. As we



**Figure 1** P-S6 immunostaining patterns in normal vulvar epithelium and carcinoma. H&E staining (top) and p-S6(S235/236) immunostaining (bottom) of (a) normal vulvar epithelium from case 10, (b) a carcinoma that was associated with a classic VIN, case 14, (c) a carcinoma that was associated with a differentiated VIN, case 1. Bar in a is 200  $\mu$ m. Note that p-S6 positive cells are confined to terminally differentiated suprabasal layers in the normal epithelium shown in a, whereas basal and parabasal cells are p-S6 positive in many areas of the carcinomas shown in b and c.

found previously for normal oral epithelium, the basal cells and first several suprabasal cell layers of normal vulvar epithelium were p-S6 negative, with more superficial terminally differentiated cells variably positive (Figure 1a). In contrast, p-S6 positive basal cells were frequent in all 12 carcinomas present in our specimen set (Figures 1b and c).

As summarized in Table 1, p-S6 was detectable in basal cells of seven of the nine classic VINs (Figures 2a–c) and in all seven differentiated VINs examined (Figures 2d–f). This result was consistent with our recent analysis of oral dysplasias.<sup>29</sup> P-S6 positive regions of VINs typically showed full-thickness cytoplasmic staining (Figure 2). Regions identified as dysplastic (VIN) by conventional H&E histopathologic morphology in each specimen were analyzed for the percentage of the dysplastic region that contained p-S6 positive basal cells. This ranged from 15 to 65% for differentiated VINs and from 0 to 60% for classic VINs (Table 1).

### P-S6 in Nondysplastic Vulvar Epithelial Lesions

It is widely believed that vulvar carcinoma arises with increased probability from lichen sclerosis lesions, which may not display apparent dysplasia or atypia.<sup>11–14</sup> In our survey of the 16 VINs described above, we found p-S6-positive basal cells in a region of lichen sclerosis without morphologic atypia adjacent to a differentiated VIN and invasive carcinoma in case 5 (data not shown). This observation prompted us to examine an additional case of lichen sclerosis associated with carcinoma plus a set of 12 vulvar biopsies that had been classified histopathologically as nondysplastic and were not associated with apparent VIN or carcinoma at the time of biopsy. These included six lichen sclerosis lesions and six hyperplastic and/or inflamed lesions (Table 2).

As we had found for wounds and ulcers in nondysplastic oral epithelium,<sup>29</sup> basal cells at the edge of a wound in histologically normal vulvar epithelium were p-S6 positive (Figure 3a). (In this connection, it is important to note that in our analysis of p-S6 staining of VINs above we did not score as positive any regions that were within 20 cells of the edge of a wound or ulcer). Ulcerated regions of two inflamed, non-dysplastic lesions also contained basal p-S6 positive cells (Table 2, Figure 3b). The other hyperplastic and inflamed lesions were basal p-S6 negative (Table 2, Figure 3c).

The lichen sclerosis lesion of a patient that had carcinoma at the time of biopsy had an extensive region of basal p-S6 positive cells (Figure 3d), as did one of the six non-carcinoma-associated lichen sclerosis lesions (Figure 3e). The other lichen sclerosis lesions examined were basal p-S6 negative (Figure 3f). Importantly, regions of lichen sclerosis lesions in which the epithelium had separated from the underlying connective tissue, as commonly seen

**Table 1** Summary of immunostaining results for VIN lesions

Case #	Patient age (years)	Histopathologic classification	VIN length in mm, (% with basal p-S6)	Lam $\gamma^2$	Tumor in specimen
1	81	dVIN	20 (65)	P	SCC 2
2	82	dVIN	4.2 (60)	ND	SCC 2
3	55	dVIN	10 (60)	N	SCC
4	69	dVIN	35 (25)	P	SCC 1
5	ND	dVIN with lichen sclerosis	7.5 (65)	N	SCC
6	ND	dVIN	18 (15)	N	No tumor
7	65	dVIN	40 (50)	P	SCC
8	65	cVIN3	10 (60)	P	No tumor
9	52	cVIN3	28 (35)	P	SCC
10	56	cVIN3	18 (5)	N	SCC
11	93	cVIN3	30 (60)	N	SCC sup.
12	ND	cVIN3	19 (0)	N	No tumor
13	ND	cVIN3	30 (60)	ND	SCC
14	ND	cVIN2/3	27 (0)	ND	SCC
15	ND	cVIN2	10 (5)	ND	No tumor
16	ND	cVIN3	8 (12)	N	SCC sup.

Abbreviations: cVIN, classic VIN; dVIN, differentiated VIN; N, no laminin  $\gamma^2$  positive cells; ND, not determined; P, some laminin  $\gamma^2$  positive cells; SCC, squamous cell carcinoma; sup, superficially invasive.

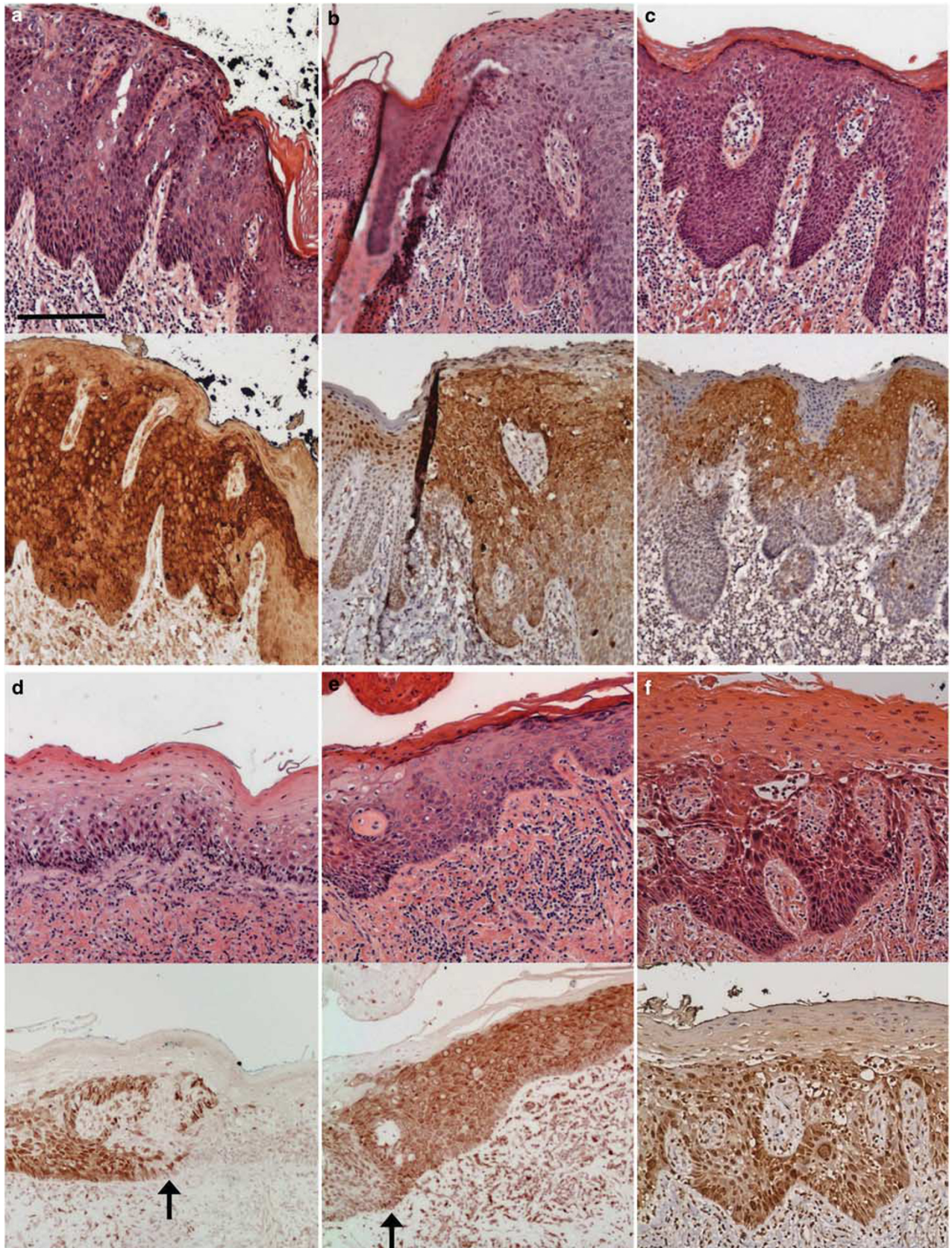
Sections were examined microscopically to determine the total 'length' of dysplastic areas and the percent of the dysplastic regions that were basal layer p-S6 positive. Classic VINs and carcinomas were classified as grades 1, 2, or 3 according to their degree of differentiation as assessed by morphologic criteria.<sup>41</sup>

in lichen sclerosis, were p-S6 negative (Figure 3f), indicating that this did not trigger a wound-related p-S6 response. In all three cases of basal p-S6 positive lichen sclerosis, the suprabasal cells were not stained, different from the basal-plus-parabasal or full-thickness p-S6 staining typical for differentiated VIN lesions (Figure 2). We concluded from these results that neither hyperplasia nor inflammation in the absence of cellular atypia triggers abnormal, basal p-S6. However, a minority of lichen sclerosis lesions without atypia exhibits abnormal p-S6 staining, biased in our small sample toward lesions associated with carcinoma at time of biopsy.

### The Relation of Laminin $\gamma^2$ Overexpression to Basal p-S6 Immunostaining

Laminin  $\gamma^2$  is not detectable immunohistochemically in the basal cells of normal epithelium but frequently is expressed abnormally by cells in the basal layer of invading fronts of many carcinomas<sup>21–23</sup> and also by some basal cells in many oral and interfollicular epidermal dysplasias.<sup>24,25,29</sup> Our recent study of oral dysplasias found that laminin  $\gamma^2$  overexpression occurs solely within regions that immunostain for p-S6(ser235/236) in the basal layer.<sup>29</sup> We therefore asked whether laminin  $\gamma^2$  expression is linked to basal cell S6 phosphorylation in vulvar lesions. As shown in Figures 4a and b, laminin  $\gamma^2$  was detectable in some basal cells of two of the six differentiated VINs and five of





**Figure 2** P-S6 immunostaining in VINs. H&E (top) and p-S6(S235/236) (bottom) staining of classic VIN cases 8 (a), 13 (b), and 16 (c) and of differentiated VIN cases 7 (d), 2 (e), and 6 (f). Bar in a is 200  $\mu$ m. Note full-thickness p-S6 staining in the dysplastic region at left in a, adjacent to a region with normal p-S6 staining. Note periodic short stretches of basal and parabasal p-S6 positive cells in panels b, c and the abrupt margin (indicated by arrows) between basal p-S6 positive and negative regions in the dysplasias shown in d, e.



**Table 2** Summary of immunostaining results for nondysplastic vulvar lesions

Case #	Histopathologic classification	Basal layer p-S6
17	Hyperpl, inflamm, ulcer	N (ulcer P)
18	Hyperpl, inflamm	N
19	Lichen sclerosus	N
20	Lichen sclerosus	N
21	Early lichen sclerosus	N
22	Hyperpl	N
23	Hyperpl	N
24	Yeast infection, severe inflamm, ulcer	N (ulcer P)
25	Normal	N
26	Early lichen sclerosus/mild dysplasia*	N
27	Lichen sclerosus	N
28	Lichen sclerosus, hyperpl	P
29	Lichen sclerosus (assoc. with SCC)	P

Abbreviations: Hyperpl, hyperplasia; inflamm, inflammation; N, no p-S6-positive basal cells; P, some p-S6-positive basal cells; SCC, squamous cell carcinoma; ulcer, specimen contained an area lacking complete epithelial coverage.

Asterisk for case 26 indicates that the pathologist who originally evaluated the lesion classified it as mild dysplasia, whereas one of us (C.P.C.) detected no dysplasia in the series of sections cut from the block for H&E and immunohistochemical staining.

the nine classic VINs tested, as well as in some areas of all invasive carcinomas (Figure 4c). As we had found for oral dysplasias,<sup>29</sup> more cells in VINs were p-S6 positive than were laminin  $\gamma^2$  positive. When present, laminin  $\gamma^2$  positive cells always lay within regions of p-S6 positive basal cells (Figure 4), suggesting that events resulting in S6 phosphorylation are necessary preconditions for laminin  $\gamma^2$  overexpression.

## Discussion

The results we have described here demonstrate that abnormal phosphorylation of ribosomal protein S6 in basal epithelial cells is a readily detectable and reliable immunohistochemical biomarker of differentiated VIN lesions. Consistent with our recent findings about oral dysplasias,<sup>29</sup> p-S6(S235/236) promises to be a very useful, single biomarker for detecting non-papillomavirus-related, potential precursors of vulvar carcinoma. Unlike classic VINs, differentiated VINs lack a consistent signature of morphologic abnormalities, so an objective biomarker would be very valuable for their diagnosis.

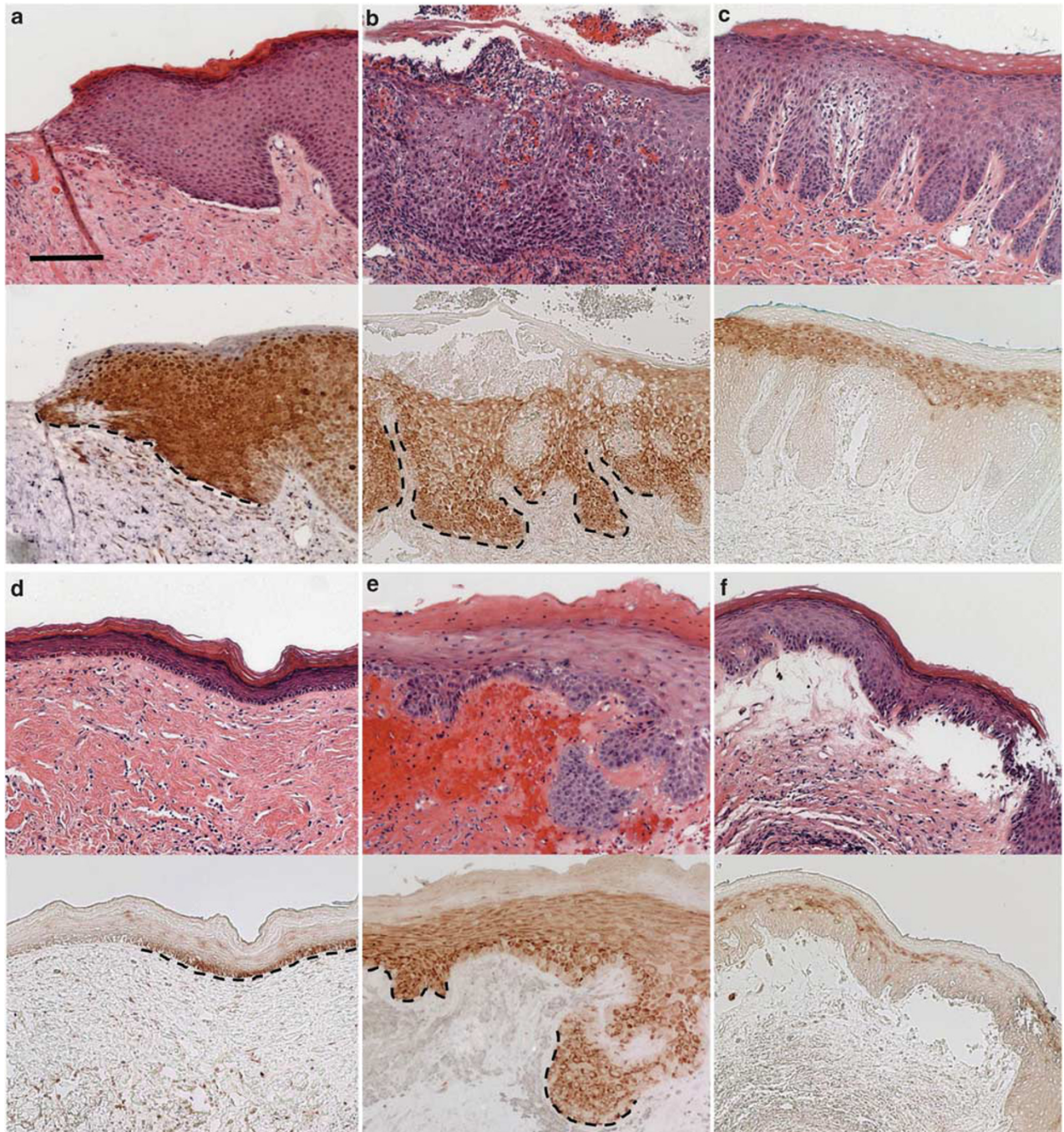
Squamous cell carcinomas typically have hyperactive EGFR/ERK and PI3K/AKT/mTOR signaling pathways.<sup>26–28,31</sup> One consequence of activation of these pathways is phosphorylation of the ribosomal protein S6. S6 is a component of the 40S ribosomal subunit and is thought to have a role in modulating efficiency of mRNA translation initiation.<sup>32</sup> S6 is activated by phosphorylation at several serine residues (S235, S236, S240, and S244) near its C-terminus.<sup>33</sup> Phosphorylation at S235 and S236 in

normal keratinocytes and squamous cell carcinoma cells is accomplished by the kinase p90RSK, which is activated by the EGFR/ERK signal pathway, whereas the S240 and S244 phosphorylations appear to be accomplished exclusively by S6K1, a kinase activated by the PI3K/AKT/mTOR signaling pathway.<sup>29,34–36</sup> An antibody specific for p-S6(S235/236) works well as an immunohistochemical staining reagent, whereas antibodies specific for the phosphorylated, activated states of other proteins in the EGFR/ERK and PI3K/mTOR signal pathways, which work well for western blotting, do not identify their antigens in neoplastic basal epithelial cells in formalin-fixed, paraffin-embedded tissue.<sup>29</sup> Therefore, p-S6(S235/236) immunostaining provides the best read-out of EGFR/ERK signal pathway hyperactivity in pathology tissue specimens.<sup>29</sup>

Laminin  $\gamma^2$  overexpression is a well-established immunohistologic marker of carcinoma<sup>21–23</sup> and occurs also in many premalignant dysplasias of oral epithelium and epidermis.<sup>24,25,29</sup> Recently, we reported that laminin  $\gamma^2$  overexpression in premalignant and malignant oral and epidermal keratinocytes occurs at the level of translation and is caused by hyperactivation of the EGFR/ERK/RSK pathway, which activates both S6 and the translation initiation factor eIF4B.<sup>29</sup> That study found that laminin  $\gamma^2$  overexpression occurs solely within regions of oral dysplasias having elevated p-S6(S235/236) levels in their basal cell layer.<sup>29</sup> The present study showed that this also is the case in VINs. P-S6 staining of VINs was more extensive than that of laminin  $\gamma^2$  and also is a direct consequence of signal pathway hyperactivity, suggesting that p-S6 is potentially more sensitive and valuable as an early biomarker of neoplasia than is laminin  $\gamma^2$ . An additional advantage of p-S6 over laminin  $\gamma^2$  is that p-S6 immunostaining does not appear to be diminished or abolished by the common histopathology service lab practice of 'baking' paraffin section slides at 50 °C, unlike the great sensitivity of laminin  $\gamma^2$  to such treatment.<sup>24,29</sup>

Biomarkers for detecting precursor lesions of vulvar squamous cell carcinoma have long been sought, especially for the often difficult to identify non-papillomavirus-related, differentiated VINs. p16<sup>INK4A</sup> is a very useful marker for diagnosing high-risk papillomavirus-related epithelial neoplasias, such as virtually all cases of cervical intraepithelial neoplasia, cervical carcinoma, and classic VIN. Full or near full thickness p16<sup>INK4A</sup> immunostaining characterizes most classic VINs, but normal and hyperplastic vulvar epidermis, lichen sclerosus, and differentiated VIN are usually p16 negative.<sup>17,18</sup> Presence of strongly p53-positive suprabasal cells was proposed as an aid for the diagnosis of differentiated VIN<sup>20</sup> but this marker is not considered highly sensitive. Independent studies found a range of 54 to 83% of differentiated VINs to have abnormal p53 immunostaining



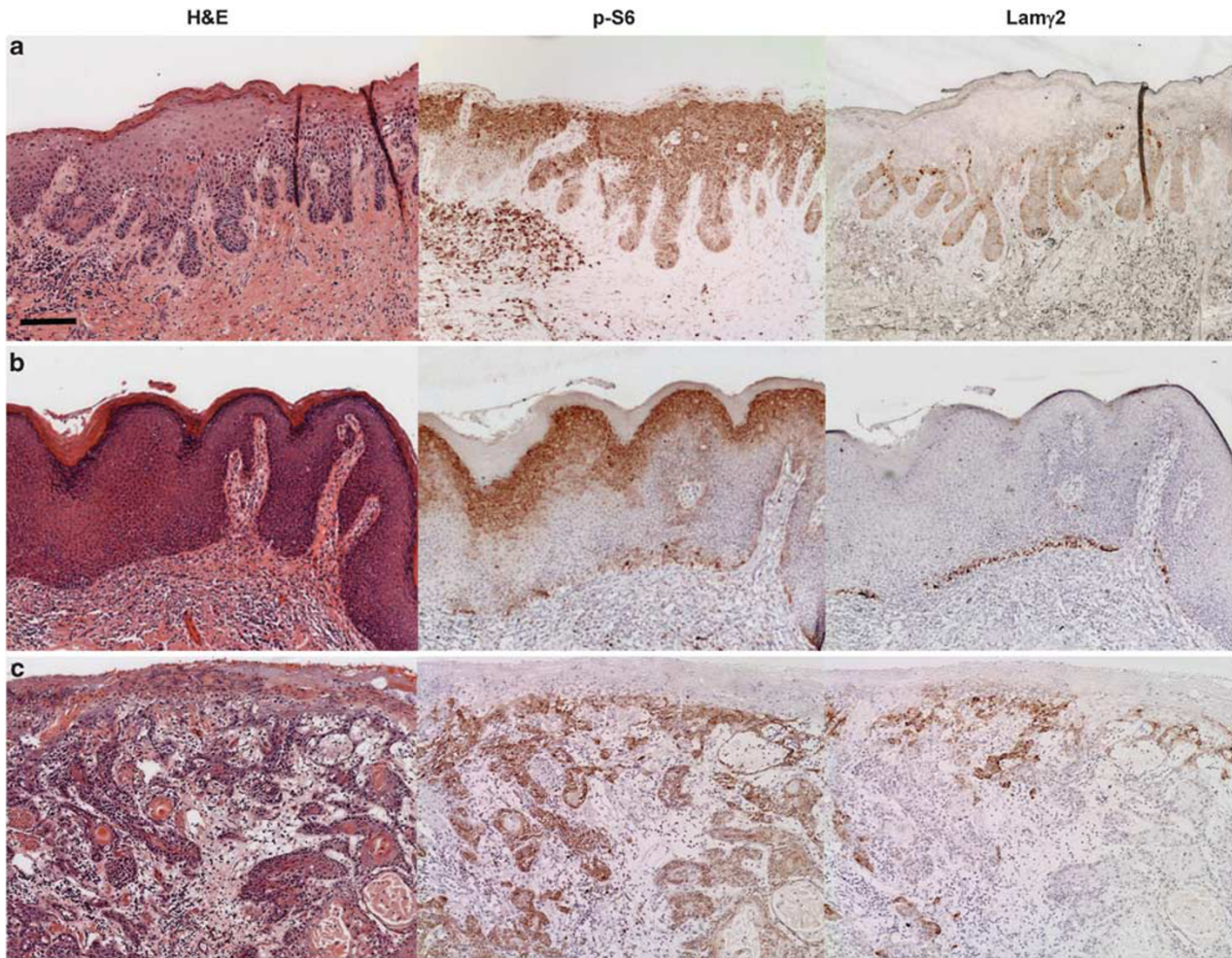


**Figure 3** P-S6 positive basal cells in settings without morphologic dysplasia. H&E staining (top) and p-S6(S235/236) immunostaining (bottom) of (a) ulcer edge in a histologically normal region of case 14, (b) severely inflamed and ulcerated region in case 24, (c) hyperplasia case 23, (d) lichen sclerosis case 29, (e) lichen sclerosis with hyperplasia case 28, (f) lichen sclerosis case 27. Bar in a is 200  $\mu$ m. Dashed lines demarcate basal p-S6-positive regions. Note p-S6 positive basal cells at wound edge in a and in ulcerated region of c, consistent with our previous findings for wounds and ulcers in normal epidermis and oral epithelium.<sup>29,40</sup> Note absence of apparent morphologic dysplasia or atypia in both p-S6 positive and negative regions of the lichen sclerosis lesions in d–f.

patterns.<sup>9,17,20,37,38</sup> The fact that p53 is detected only in some differentiated VIN lesions might be explained by differences in the type of *TP53* gene mutation, with strong p53 immunostaining associated with dominant-negative missense mutations and absence of staining associated with *TP53* deletions.<sup>9</sup> Weak basal layer p53 staining has been

reported in some lichen simplex chronicus and lichen sclerosis lesions in the absence of basal atypia and not associated with carcinoma.<sup>20</sup> In these entities, any increase in p53 immunostaining intensity has been attributed to increased p53 protein levels associated with a stress response to ischemia or inflammation rather than to mutational change.<sup>39</sup>





**Figure 4** Relation between basal p-S6 and laminin  $\gamma^2$  overexpression in VIN and carcinoma. H&E staining (left), p-S6(S235/236) immunostaining (middle), and laminin  $\gamma^2$  immunostaining (right) of (a) differentiated VIN case 1, (b) classic VIN case 9, (c) carcinoma adjacent to differentiated VIN case 4. Bar in a is 200  $\mu$ m. Note that laminin  $\gamma^2$  positive cells in the dysplasia and carcinoma always lay within p-S6 positive regions, as reported previously for oral dysplasias and carcinomas.<sup>29</sup>

Two of the nine cases of classic VIN we studied did not have detectable basal p-S6, indicating that the distinctive morphologic changes including hyperplasia characteristic of classic VIN may precede activation of the EGFR/ERK signal pathway to a level sufficient to cause S6(S235/236) phosphorylation. Our study also found basal p-S6 positivity in regions of lichen sclerosus without atypia adjacent to invasive carcinoma and in one of six lichen sclerosus lesions without atypia that were not associated with known VIN or carcinoma at time of biopsy. This finding suggests the possibility that p-S6 staining detects premalignant change in lichen sclerosus lesions before the appearance of morphologic atypia. As lichen sclerosus may sometimes precede<sup>11,13,14</sup> and be genetically linked<sup>8</sup> to differentiated VIN, p-S6 may detect premalignant change before the appearance of morphologically detectable VIN.

In summary, our study provides evidence that p-S6(S235/236) immunostaining may be useful clinically as a biomarker of vulvar lesions at risk of

progressing to invasive carcinoma. Larger retrospective studies of VIN and lichen sclerosus lesions of patients who did or did not progress to carcinoma will determine the value of p-S6 in diagnosing vulvar premalignancy.

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### Disclosure/conflict of interest

The authors declare no conflict of interest.

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