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Differentiated vulvar intraepithelial neoplasia contains *Tp53* mutations and is genetically linked to vulvar squamous cell carcinoma

Alvaro P Pinto[1,3], Alexander Miron[2], Yosuf Yassin[2], Nicolas Monte[3], Terri YC Woo[3], Karishma K Mehra[3], Fabiola Medeiros[4] and Christopher P Crum*[,3]

[1]*Department of Pathology, Federal University of Paraná, Curitiba, PR, Brazil;* [2]*Department of Cancer Biology, Dana Farber Cancer Institute, Boston, MA, USA;* [3]*Division of Women's and Perinatal Pathology, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA and* [4]*Department of Pathology, Mayo Clinic Foundation, Rochester, NM, USA*

Differentiated vulvar intraepithelial neoplasia is a unique precursor to vulvar squamous cell carcinoma that is typically HPV-negative and frequently associated with nuclear p53 staining. These features imply a mode of pathogenesis involving somatic mutations. However, the genetic relationship of differentiated vulvar intraepithelial neoplasm and vulvar squamous cell carcinoma and the role of *Tp53* mutations in this process have not been resolved. We analyzed 11 differentiated vulvar intraepithelial neoplasms and 6 associated vulvar squamous cell carcinomas. Sections were stained for p53 and p63 and DNA from multiple epithelial sites, representing normal control tissues ($n=10$), differentiated vulvar intraepithelial neoplasias ($n=18$), and vulvar squamous cell carcinomas ($n=6$), were obtained by laser capture microdissection, and sequenced for exons 2–11 of *Tp53*. Six of 10 cases contained at least one *Tp53* mutation-positive differentiated vulvar intraepithelial neoplasia focus; 4 strongly p53 immuno-positive and 2 negative. Staining for p53 and p63 co-localized, targeting the immature epithelium, but surface epithelium was *Tp53* mutation-positive. Four of five vulvar squamous cell carcinomas were *Tp53* mutation-positive; two shared identical *Tp53* mutation with adjacent differentiated vulvar intraepithelial neoplasia. Disparate foci of differentiated vulvar intraepithelial neoplasia often showed different mutations consistent with multiple neoplastic clones. Differentiated vulvar intraepithelial neoplasia is, with few exceptions, associated with *Tp53* mutations and will be p53 immunopositive when missense mutations (*versus* some nonsense and all deletion mutations) are present. Multiple *Tp53* mutations in different sites supports the presence of multiple independent genetic events, but shared *Tp53* mutations in both differentiated vulvar intraepithelial neoplasia and vulvar squamous cell carcinoma support a genetic relationship between the two. The confinement of p53 staining to immature cell nuclei is consistent with maturation-dependent degradation of mutant p53 protein.
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Mutation of the tumor suppressor *Tp53* gene is the most common cancer-related genetic change in humans.[1] In the lower female genital tract, it is uncommon in the cervix, in which human papillomavirus (HPV) is the causative agent in more than 90% of tumors. In the vulva, approximately 40% of malignancies can be linked to HPV.[2] In this HPV-mediated pathway, E6 and E7 viral oncoproteins bind to host regulatory proteins, presumably leading to degradation of p53 protein and inactivation of the retinoblastoma gene protein Rb, two essential tumor suppressor gene products.[3] These interactions, enhanced by the viral genome integration, lead to a deregulation of the cell cycle that is manifested by an abnormal expression of cell cycle-associated proteins, among them p16, a cyclin-dependent kinase inhibitor that downregulates progression

*Correspondence: Dr CP Crum, MD, Women's and Perinatal Pathology Division, Department of Pathology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA.
E-mail: ccrum@partners.org
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through the G1-S transition checkpoint of the cell cycle.[4,5] As a consequence, classic vulvar intraepithelial neoplasias are typically diffusely $p16^{ink4}$ positive. In contrast, approximately 60% of vulvar carcinomas are not associated with HPV and are often coupled to a precursor lesion designated simplex or differentiated vulvar intraepithelial neoplasias.[2,6] As a result of their mature appearance and lack of the conspicuous atypia attributed to classic vulvar intraepithelial neoplasias, differentiated vulvar intraepithelial neoplasias are not as well defined. They typically develop in association with lichen sclerosus and lichen simplex chronicus. Previous studies had revealed the presence of *Tp53* mutations in some of these tumors.[7–9] Clinicopathological overlap between HPV-associated and non-HPV-associated tumor may be observed in some cases, in which immunohistochemistry with p16 and HPV testing are more reliable than morphology for classification of both vulvar intraepithelial neoplasia and vulvar squamous cell carcinoma.[10,11]

In the HPV-related pathway, classic VIN is generally accepted as a precursor of invasive carcinoma. Although differentiated vulvar intraepithelial neoplasia is a likely candidate for precursor of HPV-negative vulvar squamous cell carcinoma and has been implied by strong p53 immunohistochemical signal, the genetic relationship between these two entities is not well understood because of molecular heterogeneity in both cancers and precursors.[12–15] In this study, we aim to (1) determine whether differentiated vulvar intraepithelial neoplasias contain *Tp53* mutations, (2) correlate *Tp53* mutation status with p53 immunostaining, and (3) establish whether differentiated vulvar intraepithelial neoplasias and vulvar squamous cell carcinomas share identical *Tp53* mutations.

Materials and methods

Case Selection

Eleven consecutive cases from 2005 to 2008 originally diagnosed as differentiated vulvar intraepithelial neoplasia were selected. Specimens ranged from biopsies to radical surgical products and in six cases the vulvar intraepithelial neoplasias were associated with vulvar squamous cell carcinoma.

Immunohistochemistry

Cases were sectioned and stained for p53 and $p16^{INK4}$. Four-micrometer sections of formalin-fixed, paraffin-embedded tissue were cut and placed on glass slides. Tissue sections were de-paraffinized and rehydrated through xylene and graded alcohols. Endogenous peroxidase activity was blocked by incubation in a solution of 3% hydrogen peroxide in 100% alcohol (1:1). Heat-induced antigen retrieval was carried out with Dako Target Retrieval Solution, pH 6.0, and pressure cooker heated (122–125 °C) for 30 s at 15–24 p.s.i. Sections were incubated 40 min at room temperature with antibodies directed against p53 (clone: E6H4, MTM laboratories AG, Heidelberg, Germany) at a 1:1200 dilution, and against $p16^{INK4}$ (clone 16P07, NeoMarkers Fremont, CA, USA) at an unknown concentration (pre-diluted). Application of the primary antibodies was followed by incubation 30 min with Dako Labeled Polymer-HRP anti-mouse IgG as a secondary antibody, and visualized with 3, 3′–diaminobenzidine (DAB) as a chromogen (Envision + System) and Mayer hematoxylin counterstaining. Sections containing normal epithelia were included in each run to assess background. Positive slide controls containing colon adenocarcinoma (for p53) and cervical intraepithelial neoplasia (for $p16^{INK4}$) were included in each staining run.

In addition, serial sections from a small series of p53-positive differentiated vulvar intraepithelial neoplasias, vulvar squamous cell carcinomas, and normal mucosa were stained for p63, a marker of basal or immature squamous cells that has been linked to stem cell characteristics.[16] The purpose was to determine the limits of p53 immunostaining in these epithelia using an objective marker for cell differentiation. Immunostaining was performed with the 4A4 clone of p63 (courtesy of Frank D McKeon), as described earlier.[17]

HPV Detection

All 12 cases were analyzed for HPV DNA. The detection was carried out using polymerase chain reaction (PCR) on target DNA extracted from formalin-fixed, paraffin-embedded tissue samples. Briefly, 3 to 10 eight-micron sections of paraffin-embedded tissue were placed in a microcentrifuge tube and incubated with Proteinase K in a digestion buffer overnight at 62 °C. After the addition of Chelex (Bio-Rad Laboratories, CA, USA), specimens were boiled and centrifuged, and the aqueous phase was removed for testing.

The HPV DNA was detected using 16E7A and 16E7B primers.[18] These primers were designed to detect HPV 16 E7 DNA, which is found in the majority of HPV-positive vulvar intraepithelial neoplasias. These primers detect a 224-bp section of the E7 region of the HPV DNA. Amplifications were carried out under the following conditions: Initial denature of 2 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension of 72 °C for 5 min. Reaction products were run on 5% Tris/Boric Acid/EDTA (TBE) precast acrylamide gels (Bio-Rad Laboratories) at 120 V for 50 min. The gels were then stained with ethidium bromide mixed in 1 × TBE for 15 min and then washed with water. Gel images were acquired using UV Light Chamber (Bioimaging Systems).

Slide Analysis, Sample Targets, and Laser Capture Microdissection

H&E, p53, and $p16^{INK4A}$ stained slides were reviewed by two pathologists (APP and CPC). The immunoreactivity of p53 and $p16^{INK4A}$ in vulvar intraepithelial neoplasia lesions and when present, in their adjacent vulvar squamous cell carcinoma, was scored as positive or negative based on the localization and extent within the epithelium. A positive score for p53 required strong nuclear staining that obscured nuclear detail. Positive cases were further classified according to stain location as (1) when restricted to the basal layer, and (2) when present in supra-basal layers. A positive score for p16 required strong and diffuse (more than 90% of the lesion) nuclear and cytoplasmic staining. Positive cases were sub-classified as (1) when signal was confined to the lower half of the epithelium, and (2) when it was trans-epithelial. Fields for laser microdissection were selected based on H&E and p53 stains and included multiple tissue sites representing normal controls (three lymphoid aggregates and seven squamous epithelial), differentiated vulvar intraepithelial neoplasia ($n=18$), and vulvar squamous cell carcinoma ($n=6$). Two p53 immunohistochemistry-positive cases (#s 4 and 6) had the upper and lower regions of the differentiated VIN epithelium individually microdissected. Other two cases (#s 2 and 5), had in addition, the peripheral and central regions of its associated tumor nests individually microdissected based on the p53 expression.

Laser Capture Microdissection and DNA Extraction

Sections (8 μm) for laser capture microdissection were cut and placed on glass slides coated with PEN membrane (Arcturus Bioscience). A section of each specimen was then stained with H&E and examined microscopically to confirm the diagnosis. Targeted areas were selectively microdissected using the PALM microbeam instrument (Carl Zeiss Microimaging, Munich, Germany) according to standard protocols. Cells were captured on a thin film attached to a plastic cap, which was then attached to an Eppendorf tube containing 50 AL digestion buffer (40 mMol/l Tris-HCl (pH 8.0), 1 mmol/l EDTA, 0.5% Tween 20, and 0.5 Ag/AL proteinase K) and incubated at 37 °C in inverted position for 24 to 48 h. After a brief spin at 1000 r.p.m., the supernatant was transferred into a 1.5-ml centrifuge tube. The proteinase K was inactivated at 95 °C for 10 min and the solution was kept at −20 °C in aliquot.

Analysis for *Tp53* Mutations

All samples were analyzed for *Tp53* mutations. Genomic DNA was amplified by PCR using tailed primers designed to amplify exons 2 to 11 of *Tp53*. A secondary amplification was performed using primers specific to the tail sequences used in the primary amplification. PCR products were then sequenced from both strands using the same tailed primers. Data were analyzed using the Mutation Surveyor program (Soft Genetics, State College, PA, USA). Candidate mutations identified using the software was compared with the Universal Mutation Database (http://www.umd.be:2072/IFAMTP53A.shtml) for cancer-associated *Tp53* mutations.

As formalin fixation can introduce spurious mutations into somatic DNA, all *Tp53* mutation-positive exons were re-sequenced from a replicate PCR-amplified product. Samples were scored as positive for *Tp53* mutation only if an identical mutation was identified in products of both amplifications.[19]

Results

Most Differentiated Vulvar Intraepithelial Neoplasias are Associated with *Tp53* Mutations

Micro-dissected vulvar intraepithelial neoplasia areas of the 12 cases, *Tp53* mutations and their effects in translation, immunostain (p53 and $p16^{INK4A}$) results, and HPV status were summarized in Table 1. In 6 out of 10 cases of differentiated vulvar intraepithelial neoplasia, at least one differentiated vulvar intraepithelial neoplasia focus contained one or more *Tp53* mutations.

Differentiated Vulvar Intraepithelial Neoplasias with Missense Mutations are p53 Immunohistochemistry Positive

Of the six cases with *Tp53* mutations, four were p53 immuno-positive and two were negative. In all four p53 immunohistochemistry positive, the mutation effect was either missense or splice, while deletions were found in the other two p53 immunohistochemistry-negative differentiated vulvar intraepithelial neoplasias. Case images were illustrated in Figure 1. An example of differentiated vulvar intraepithelial neoplasia that was p16 negative, p53 strongly positive, and showed the same mutation in three different micro-dissected areas is shown in Figures 1a–c. In contrast, Figure 1d shows a strong, trans-epithelial p16 signal in a case of classic vulvar intraepithelial neoplasia included for comparison.

Multiple *Tp53* Mutations Occur in Some Cases, but Common Mutations can be Identified in Differentiated Vulvar Intraepithelial Neoplasia and Adjacent Vulvar Squamous Cell Carcinomas

Base changes that occurred in the differentiated vulvar intraepithelial neoplasias and their associated vulvar squamous cell carcinomas, as well as the p53 immunostain results and *Tp53* mutation effects observed in the vulvar squamous cell carcinomas are illustrated in Table 2. The mutations

Table 1 p53 mutational analysis of dVIN

Case	*Target*	*Nucleotide change*	*Amino acid change*	*Mutation effect*	*p53*	*p16*	*HPV*
1	dVIN	Amplification failed	—	—	P2	P1	N
2	dVIN (1)	817C>CT, IVS10+1G>GC	ARG>CYS, NA	Missense, splice	P2	N	N
	dVIN (2)	817C>CT	ARG>CYS	Missense			
3	dVIN	817C>CT, 844C>CT	ARG>CYS, ARG>TRP	Missense, Missense	P2	N	N
4	dVIN (1)	743G>GA	ARG>GLN	Missense	P2	N	N
	dVIN (2)	743G>GA	ARG>GLN	Missense			
	dVIN (3)	743G>GA	ARG>GLN	Missense			
5	dVIN (1)	415A>AT, 746G>GA	LYS>STOP,ARG>LYS	Nonsense, missense	P2	N	N
	dVIN (2)	415A>AT, 746G>GA	LYS>STOP,ARG>LYS	Nonsense, missense			
	dVIN (3)	746G>GA	ARG>LYS	Missense			
6	dVIN	None	—	—	P1	P1	N
7	dVIN	None	—	—	N	N	N
8	dVIN	896_909del14	NA	Deletion	N	N	N
9	dVIN (1)	896_909del14	NA	Deletion	N	N	N
	dVIN (2)	595G>GA, 896_909del14	NA	Deletion			
10	dVIN	None	—	—	N	N	N
11	dVIN	None	—	—	N	N	N

N: negative; NA: not applicable; P: positive; P1 for p53: restricted to the basal layer; P2 for p53: extended to supra-basal layers; P1 for p16: restricted to the lower half of the epithelium; P2 for p16: trans-epithelial.

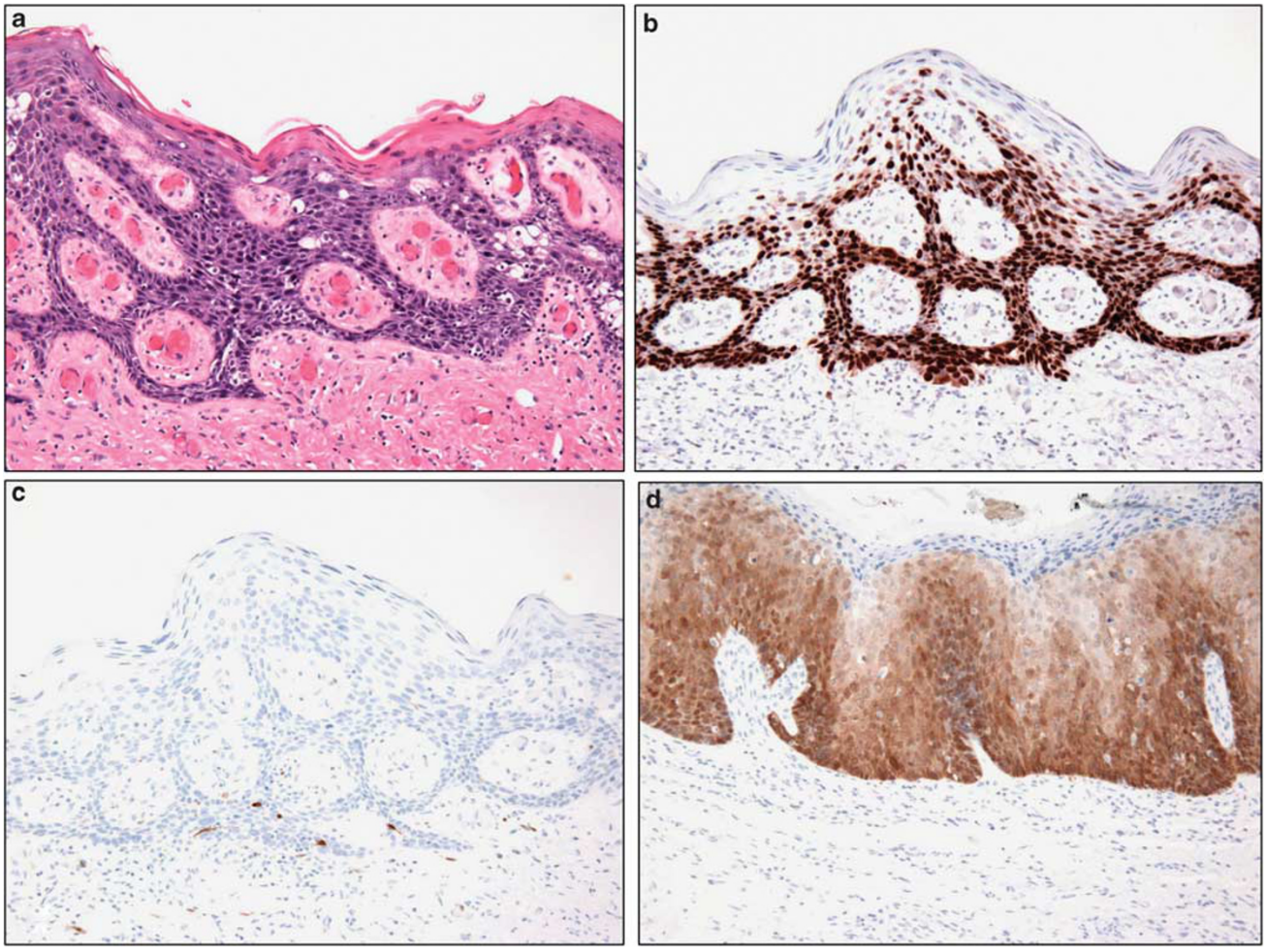

Figure 1 An example of differentiated vulvar intraepithelial neoplasia (case #4) and a classic vulvar intraepithelial neoplasia for comparison: (**a**) H&E shows differentiated vulvar intraepithelial neoplasia with parakeratosis and elongated anastomotic rete ridges containing mild nuclear atypia; (**b**) strong p53-positive staining in the lower layers of the epithelium in differentiated vulvar intraepithelial neoplasia; (**c**) absence of p16 signal in differentiated vulvar intraepithelial neoplasia; (**d**) p16 trans-epithelial staining in a classic vulvar intraepithelial neoplasia for comparison. Original magnifications: ×200.

Table 2 p53 mutational analysis of dVIN and co-existing squamous cell carcinoma (VSCC)

Case	*Area dissected*	*VIN nucleotide change*	*VIN p53*	*VSCC nucleotide change*	*VSCC mutation effect*	*VSCC p53*
2	dVIN (1)	IVS10+1G>GC	P	IVS10+1G>GC	Missense, splice	P
	dVIN (2)	817C>CT		817C>CT		
		386C>CT		692C>CT		
		817C>CT		746G>GA		
5	dVIN (1)	746G>GA, c.415A>AT	P	746G>GA	Missense	P
	dVIN (2)	746G>GA, c.415A>AT				
	dVIN (3)	746G>GA				
7	dVIN	None	N	981T>G	Nonsense	N
10	dVIN	None	N	637C>T	Nonsense	N
11	dVIN	None	N	None	—	N

N: negative; P: positive.

found in either differentiated vulvar intraepithelial neoplasias or vulvar squamous cell carcinomas were distributed predominantly (70%) throughout exons 5 to 9. Four out of five differentiated vulvar intraepithelial neoplasia-associated carcinomas were *Tp53* mutation-positive. From those, two contained missense mutations and were p53 immunohistochemistry positive. The two p53 immunohistochemistry-negative vulvar squamous cell carcinoma presented with nonsense mutations. In two of four differentiated vulvar intraepithelial neoplasia/vulvar squamous cell carcinoma cases, both entities shared an identical *Tp53* mutation. However, disparate foci of differentiated vulvar intraepithelial neoplasia often showed different mutations consistent with multiple neoplastic clones. Figure 2 illustrates a case with differentiated vulvar intraepithelial neoplasias dissected from three distinctive areas plus the associated vulvar squamous cell carcinoma that showed strong positivity for p53, as well as the same *Tp53* mutation (Table 2).

In the p53 immunohistochemistry-positive differentiated vulvar intraepithelial neoplasias in which the basal cells were positive, *Tp53* sequence analysis of microdissected regions showed the following: (1) identical mutations in three out of three areas of differentiated vulvar intraepithelial neoplasia in case #4, and in two out of three areas of differentiated vulvar intraepithelial neoplasia and in one area of invasive vulvar squamous cell carcinoma in case #5; (2) disparate mutations and disparate plus similar mutations were identified in two areas of differentiated vulvar intraepithelial neoplasia and one area of vulvar squamous cell carcinoma, respectively, in case #2 (3) absence of *Tp53* mutations in the differentiated vulvar intraepithelial neoplasia of case #6.

Expression of Mutated p53 Protein, when Detected, is Confined to the Immature Keratinocytes and Degraded as a Function of Keratinocyte Maturation

As p53 staining is confined to the basal-type cells in differentiated vulvar intraepithelial neoplasia, epithelial foci from the lower and upper layers were separately microdissected and sequenced. Samples derived from the higher p53 immunohistochemistry-negative epithelial layers of one out of two areas of differentiated vulvar intraepithelial neoplasia in case #2 and two out of three areas of differentiated vulvar intraepithelial neoplasia in case #4 were also mutation positive, consistent with derivation from the same clone as the p53-positive basal cells. Staining with p63, a p53 homolog that is a marker for immature keratinocytes revealed an identical pattern of nuclear distribution (Figure 3). Combined with the sequencing data, this supports the concept that mutated p53 protein is degraded as a function of keratinocyte maturation.

Discussion

This study has confirmed that most differentiated vulvar intraepithelial neoplasias are associated with *Tp53* mutations or deletions, that p53 immunohistochemistry positivity is dependent on the nature of the mutation, that the vulvar mucosa is prone to the emergence of more than one *Tp53* mutation, and that in some instances, the vulvar squamous cell carcinoma is genetically linked to its presumed precursor (differentiated vulvar intraepithelial neoplasia).[7,9] Predictably, a majority (70%) of the mutations found in differentiated vulvar intraepithelial neoplasia and vulvar squamous cell carcinoma were located in exons previously shown to contain the highest incidence of mutations in vulvar squamous cell carcinoma, exons 5 through 9.[9,19,20] Four out of five differentiated vulvar intraepithelial neoplasia-associated carcinomas were *Tp53* mutation-positive.

Interpretation of p53 immunohistochemistry positivity requires some care. Immunohistochemistry over-expression of p53 has been reported in lichen simplex chronicus, lichen sclerosus, and differentiated vulvar intraepithelial neoplasia adjacent to HPV-negative vulvar cancer.[7,11,12,19,20] The immunohistochemistry signal observed in lichen simplex chronicus and lichen sclerosus is usually weak and confined to a single, un-expanded layer of basal

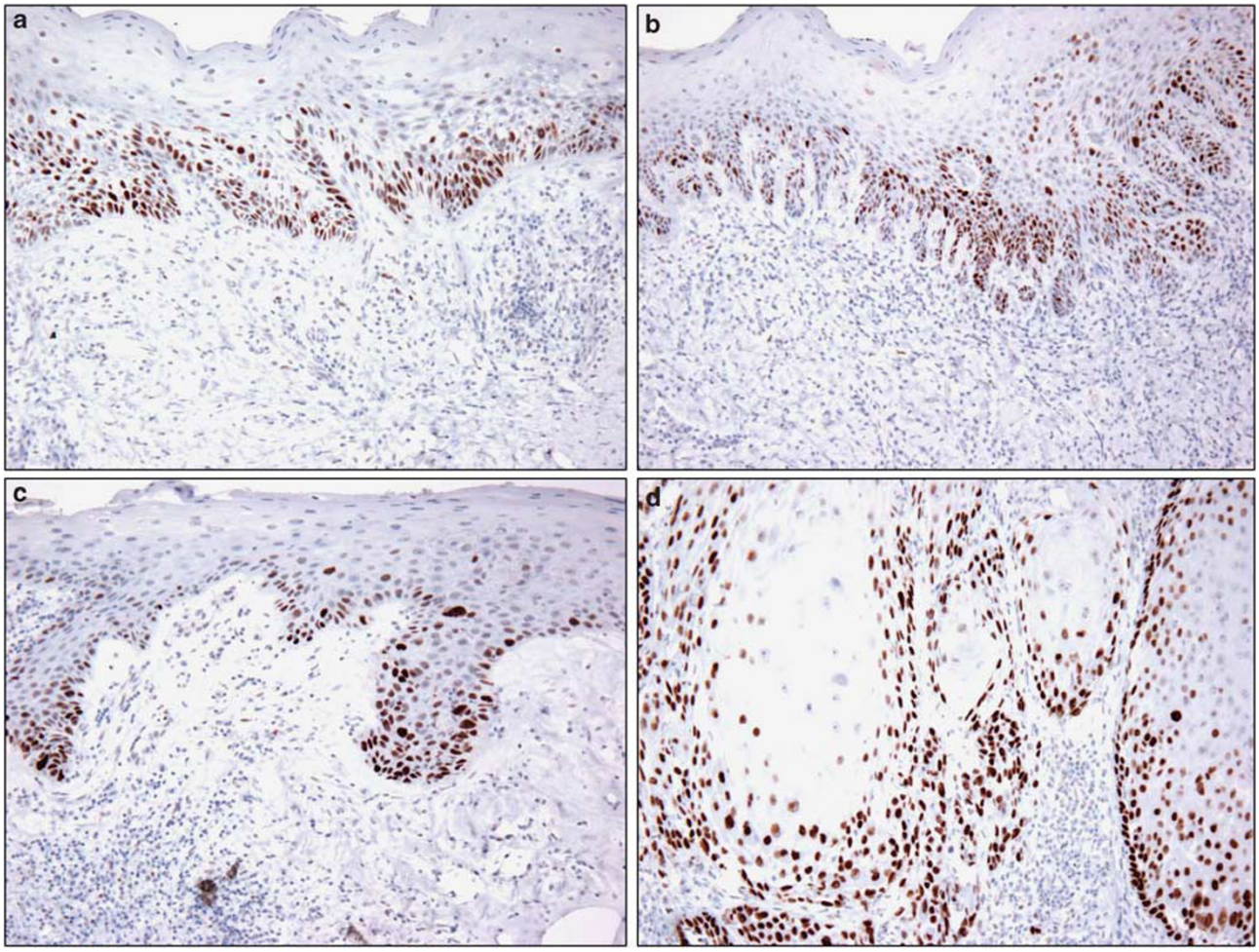

Figure 2 A case (#5) in which three differentiated vulvar intraepithelial neoplasias (**a–c**) and the associated vulvar squamous cell carcinoma (**d**) share similar p53 immunoexpression and mutations: (**a**, **b**) and (**c**) p53 staining in basal and supra-basal layers of the differentiated vulvar intraepithelial neoplasias; (**d**) p53 signal in the peripheral region of the invasive tumor. The same mutation was found in (**a**), (**b**, **c**) and (**d**). Lesions (**a**) and (**b**) shared another mutation that was not found in the tumor (**d**). Original magnifications: $\times 200$.

cells.[12,21] In these entities, *Tp53* mutations are usually not found; therefore, the p53 protein signal has been attributed to upregulation of p53 wild-type protein, possibly reflecting a stress response to inflammation or ischemia.[21] In contrast, p53 immunohistochemistry signals in differentiated vulvar intraepithelial neoplasia have been described as strong and constant, and often involving more than a single layer of basal-type cells, as observed in the majority (4/5) of our cases (Figures 1b, 2a–c).[12,22,23] The immunohistochemistry signal observed in this context has been presumed to derive from an abnormal (mutated) p53 protein. This argument is supported by: (1) a significant association of p53 immunoreactivity with loss of heterozygosity (LOH) at different chromosomal loci, including the *Tp53* locus, 17p13; and (2) the detection of *Tp53* mutations in p53 immunohistochemistry-positive atypical lichen sclerosus (or differentiated vulvar intraepithelial neoplasia).[8,19,20]

This study revealed *Tp53* mutations in most but not all differentiated vulvar intraepithelial neoplasias. The two p53 immunohistochemistry-negative cases could be explained by *Tp53* deletions that would lead to premature termination of translation, whereas the four p53 immunohistochemistry-positive cases with missense mutations were able to accumulate abnormal p53 protein. The fact that disparate foci of differentiated vulvar intraepithelial neoplasia presented with different *Tp53* mutations in our cases is consistent with the hypothesis that the vulvar epithelium is prone to multiple events leading to independent cell populations with these mutations. Evidence linking these mutations to adjacent vulvar squamous cell carcinomas has been limited. One previous study showed shared *Tp53* mutations in both lichen sclerosus and vulvar squamous cell carcinoma. Otherwise, the link between differentiated vulvar intraepithelial neoplasia and keratinizing vulvar squamous cell

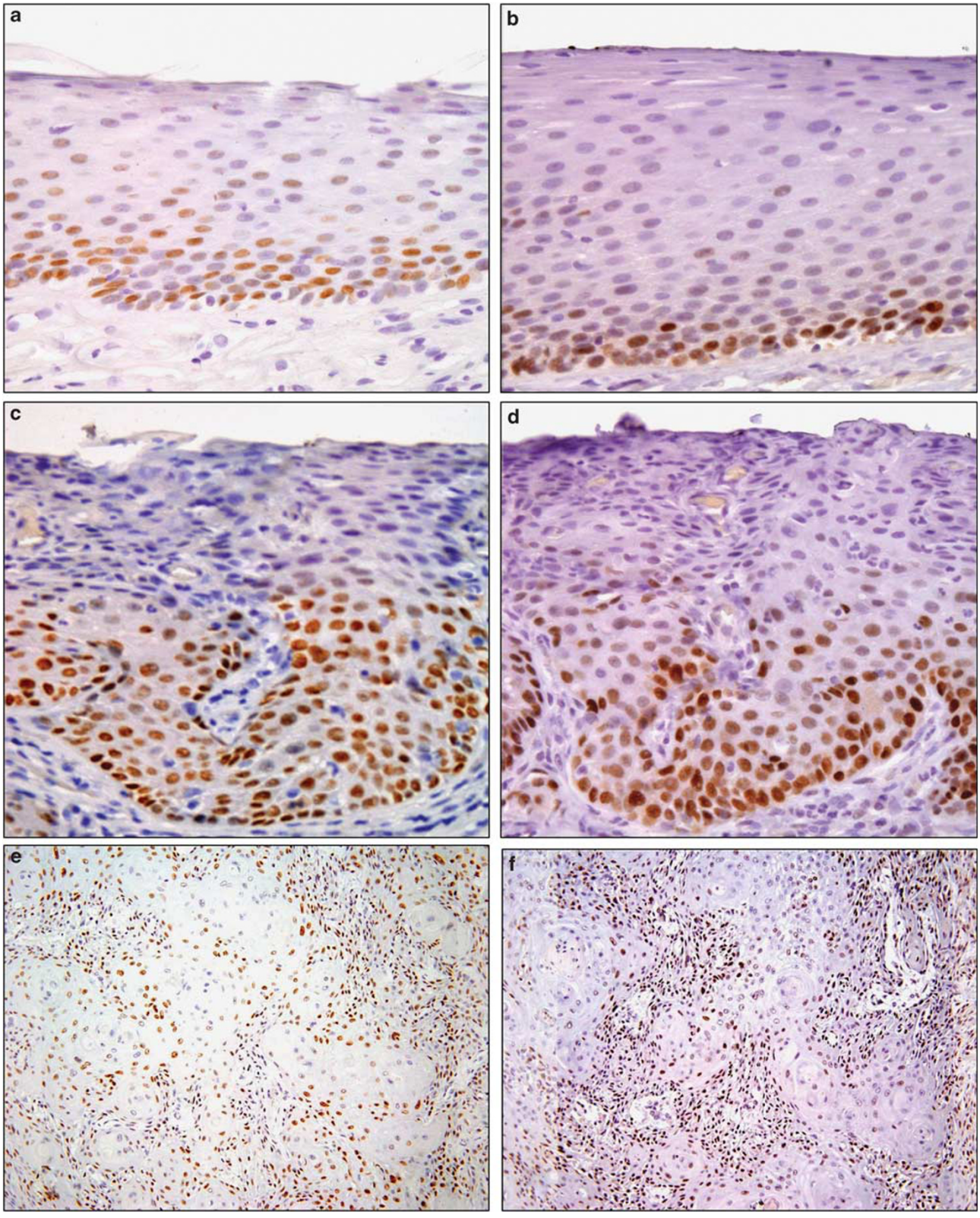

Figure 3 Co-localization of p53 (**a, c, e**) and p63 (**b, d, f**) immunostaining in normal mucosa, differentiated vulvar intraepithelial neoplasia and vulvar squamous cell carcinoma respectively.

carcinoma has been inferred from prospective follow-up studies or shared patterns of allelic loss.[24] We were able to show that coexisting differentiated vulvar intraepithelial neoplasia and vulvar squamous cell carcinoma shared the same mutation in two of our cases. Thus, some foci of differentiated vulvar

intraepithelial neoplasia represent direct cancer precursors while others are independent events, in keeping with previous studies of allelic imbalance.[13,15] It should be emphasized that although shared *Tp53* mutations alone will provide evidence of a shared cell of origin for differentiated vulvar intraepithelial neoplasia and vulvar squamous cell carcinoma, it is an oversimplification to assume that the *Tp53* mutation is the initial carcinogenic event. Vanin *et al* were able to show LOH of *Tp53* in vulvar squamous cell carcinoma and lichen sclerosus in the absence of mutations.[20] They suggested that actual *Tp53* mutations were usually late events and that the LOH affecting *Tp53* also affected another tumor suppressor gene on 17p.[20]

The presence of p53 immunopositivity in only the immature component of the differentiated vulvar intraepithelial neoplasia is in contrast to other neoplasms with p53 mutations and we were able to confirm that the same p53 mutation was present in all of the epithelial layers, consistent with a shared genetic identity. This maturation-dependent loss of p53 immunostaining indicates that, similar to p63, this mutant protein is vulnerable to degradation in maturing keratinocytes.[25] Thus, the vertical extent of p53 staining can be expected to vary according to epithelial differentiation as shown in Figures 1–3, and the number of epithelial layers showing staining alone cannot be expected to predict whether a p53 mutation is or is not present.

In summary, this study was able to establish that (1) distinct foci of differentiated vulvar intraepithelial neoplasia contain varied *Tp53* mutations consistent with multiple neoplastic clones; (2) these neoplastic foci will be p53 immunopositive when missense mutations are present, and p53 immunonegative in the absence of mutations or when deletion or nonsense mutations are present; (3) differentiated vulvar intraepithelial neoplasia and vulvar squamous cell carcinoma share identical *Tp53* mutations, supporting a pathogenetic connection between them; and (4) the restraint of p53 staining to immature cell nuclei is consistent with maturation-dependent degradation of mutant p53 protein, similar to its homolog p63.[16]

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Disclosure/conflict of interest

The authors of this paper do not currently have financial relationship with the commercial enterprise whose products were discussed in this paper.

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