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ARTICLE

Molecular characterization of invasive and in situ squamous neoplasia of the vulva and implications for morphologic diagnosis and outcome

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Abstract

Human papillomavirus (HPV)-independent vulvar squamous cell carcinoma (VSCC) is an aggressive clinical entity. Current diagnostic guidelines for premalignant lesions are ambiguous, and their molecular profile and progression events are still unclear. We selected 75 samples, from 40 patients, including 33 VSCC, 8 verrucous carcinomas (VC), 13 differentiated-type vulvar intraepithelial neoplasia (dVIN), 11 suspicious for dVIN (?dVIN), 6 differentiated exophytic vulvar intraepithelial lesions (DE-VIL), 2 vulvar acanthosis with altered differentiation (VAAD), and 2 usual-type vulvar intraepithelial neoplasia (uVIN/HSIL). Invasive and precursor lesions were matched in 29 cases. Clinical information, p16 immunohistochemistry, and mutation analysis were performed on all lesions. All dVIN, ?dVIN, DE-VIL, and VAAD were p16 negative, all uVIN/HSIL were p16 positive. In the HPV-independent group, mutations were identified in 6 genes: *TP53* ($n = 40$), *PIK3CA* ($n = 20$), *HRAS* ($n = 12$), *MET* ($n = 5$), *PTEN* ($n = 4$), and *BRAF* ($n = 1$). *TP53* mutations occurred in 73% (22/30) VSCC, 85% (11/13) dVIN, 70% (7/10) ?dVIN and no VC (0/8), DE-VIL (0/6) nor VAAD (0/2). Basal atypia was the only reliable feature of *TP53* mutations. ?dVIN lesions that were non-acanthotic and atypical but obscured by inflammation, all harbored *TP53* mutations. In lesions without *TP53* mutations, *PIK3CA* (50% VC, 33% DE-VIL, 100% VAAD, 40% VSCC) and *HRAS* (63% VC, 33% DE-VIL, 0% VAAD, 20% VSCC) mutations were found. Mutational progression from in situ to invasive was seen (7/26, 27%) and usually involved *TP53* (4/26, 15%). Cases with *TP53* and *PIK3CA* co-mutations had the worse clinical outcomes ($p < 0.001$). We recommend testing for p53 in all HPV-independent lesions suspicious for dVIN, even in the presence of marked inflammation or non-acanthotic skin, particularly when close to a margin. VC, VAAD, and DE-VIL, were almost never mutated for *TP53*, but instead often harbored *PIK3CA* and *HRAS* mutations. In VSCC, combined *TP53* and *PIK3CA* mutations may inform prognosis.

Introduction

Vulvar carcinoma represents 3–5% of all gynecologic malignancies with an annual incidence of 1–2 per 100,000, accounting for 6070 cases per year and 1280 deaths in the United States in 2019 [1, 2]. Squamous cell carcinoma (VSCC) is the most common, constituting 80–90% of malignancies at this site [1]. The 5-year overall survival (OS) varies from 86.3% when disease is localized, to 52.6% when regional lymph nodes are involved, and 22.7% for distant metastases [2]. The current mainstays of treatment are surgical resection, radiation, and/or chemotherapy [3–5]. The high-risk of severe morbidity with treatment escalation

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is a major challenge in managing vulvar carcinomas, which is magnified when patients undergo pelvic exenteration for treatment refractory or recurrent disease [6–9]. There is a need for better prognostic stratification tools and treatment strategies in this patient group to help optimize outcomes and limit complications.

There has been an effort to separate VSCC into two main categories based on their pathophysiology: human papillomavirus (HPV)-associated and HPV-independent [10–13]. HPV-associated VSCC typically occur in younger women and show basaloid and warty histomorphology. HPV-independent VSCC are seen in older women, often in association with lichen sclerosus and tend to exhibit keratinizing histomorphology [12, 14]. The HPV-independent: HPV-associated VSCC ratio varies between 0.60 and 0.83, and is expected to rise further as we see rates of HPV-associated premalignant genital lesions decrease following the widespread adoption of HPV vaccination in developed countries [15–18]. Most studies have revealed worse outcomes in HPV-independent VSCC [14, 15, 17, 19–21], similar to what is observed in squamous cell carcinomas of the head and neck region [22].

The premalignant (in situ) squamous lesions preceding VSCC are a rapidly evolving field. VSCC precursors include high-grade squamous intraepithelial lesions, otherwise known as usual-type vulvar intraepithelial neoplasia (uVIN/HSIL), which leads to HPV-associated VSCC, and differentiated vulvar intraepithelial lesion (dVIN), which leads to HPV-independent VSCC. In recent years, two new lesions termed vulvar acanthosis with altered differentiation (VAAD) and differentiated exophytic vulvar intraepithelial lesion (DE-VIL), have been raised as alternate precursor lesions to HPV-independent VSCC [23–25], with a new category of HPV-independent VIN proposed to encompass all three of these precursors i.e., dVIN, VAAD, and DE-VIL [26]. The absence of *TP53* mutations in VAAD and DE-VIL, and frequent association with verrucous carcinoma, a more indolent neoplasm, have led investigators to believe they constitute a third pathway to VSCC (p53-independent/HPV-independent), which is separate from dVIN [23–25].

While the utilization of molecular information for patient diagnosis, prognostication, and treatment planning has been fervently adopted in breast cancer, malignant gliomas, colorectal cancer, and more recently, endometrial carcinoma [11–13, 27], very few studies have explored the application of molecular information in VSCC. In this study, we examine the mutational profile of VSCC, focusing particularly on the more aggressive HPV-independent subgroup, in order to uncover any biologic, prognostic, and therapeutic insights in this understudied disease. We have also included a variety of paired squamous precursor lesions, which have not yet been clearly defined on a molecular level.

Materials and methods

Patient selection

Cases were selected from the archives of Vancouver General Hospital from 1998 to 2019. Vulvectomy specimens were reviewed and preference was given to cases with features suggestive of HPV-independent VSCC with an associated in situ lesion [13, 14]. The invasive and in situ components had to be distinct enough to be separated by macrodissection (coring). In some cases, where one of the components could not be separated, only one component was included for subsequent mutational analysis. The cohort was also enriched for DE-VIL, VAAD, and VC.

Histomorphologic review

Hematoxylin and eosin (H&E) stained slides for each case was re-reviewed and the lesions were re-classified into the following categories: conventional squamous cell carcinoma (VSCC: invasive carcinoma comprising of malignant squamous cells with variable keratinization and definite stromal invasion), verrucous carcinoma (VC: well differentiated squamous tumors with minimal cytologic atypia, bullous epithelial pegs and a broad pushing front into the stroma), differentiated-type vulvar intraepithelial neoplasia (dVIN: in situ squamous precursor lesion usually located adjacent to VSCC exhibiting moderate to marked basal nuclear atypia as well as variable degrees of hyperchromasia, karyomegaly, enlarged nucleoli, atypical mitoses, dyskeratosis, elongated and anastomosing rete ridges), usual-type vulvar intraepithelial neoplasia/high-grade squamous intraepithelial lesion (uVIN/HSIL: in situ squamous lesion exhibiting basaloid morphology, hyperchromasia, crowding, anisonucleosis), differentiated exophytic vulvar intraepithelial lesion (DE-VIL: as defined by Watkins et al. [23], in situ squamous precursor lesion exhibiting prominent acanthosis or verruciform morphology, absence of conspicuous basal atypia and abnormalities in keratinocyte differentiation such as hypogranulosis, hyperkeratosis, parakeratosis and dyskeratosis), and vulvar acanthosis with altered differentiation (VAAD: as defined by Nascimento et al. [24], triad of marked acanthosis with variable verruciform architecture, loss of the granular cell layer with superficial epithelial cell pallor and multilayered parakeratosis). Cases that morphologically appeared as DE-VIL or VC, but exhibited moderate to severe basal nuclear atypia, were upgraded to dVIN and SCC, respectively. We also included a category of in situ lesions that were suspicious

for dVIN but did not display all the classic morphologic features for a confident diagnosis. These lesions were designated under the "?dVIN" category.

Immunohistochemistry (IHC)

IHC for p16 was used to discriminate between HPV-associated and HPV-independent neoplasms, as previously described [28]. p16 IHC was scored as positive if there was diffuse block-like cytoplasmic and nuclear staining and as negative for any lesser staining (such as patchy staining or absence of staining), in accordance with the LAST (Lower Anogenital Squamous Terminology) recommendations [29].

Sequencing

Mutational analysis was performed on macrodissected formalin fixed paraffin embedded tissue using a commercial next-generation sequencing (NGS) panel that targeted 123 hotspot mutations and 17 exons in 33 known cancer-related genes (*AKT*, *ALK*, *AR*, *BRAF*, *CTNNB1*, *DDR2*, *EGFR*, *ERBB2*, *ESR1*, *FGFR1*, *FGFR2*, *GNA11*, *GNAQ*, *GNAS*, *HRAS*, *IDH1*, *IDH2*, *JAK*, *KIT*, *KRAS*, *MEK1*, *MAP2K1*, *MAP2K2*, *MET*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTCH1*, *PTEN*, *RET*, *ROS1*, *SMO*, *STK11*, *TP53*), as previously described [30]. The *TP53* status for many of these tumors have been reported previously [28]. Only variants with a minimum read depth of 500, an allelic ratio ≥5%, a base quality score ≥30, and a probability score ≥0.90 for single nucleotide changes or a quality score of ≥1000 for insertion/deletion events were reported. To ensure the areas of interest were captured, post-core hematoxylin and eosin (H&E) stained slides were reviewed.

Statistics and survival analysis

The statistical significance, when comparing frequencies of events across two different groups, was assessed using a comparison of proportions analysis [31].

We performed univariate regression analyses of overall survival (OS) and progression-free survival (PFS) using *TP53* and *PIK3CA* mutational status, age, tumor size, tumor depth, focality, presence of lichen sclerosus, surgical margins, lymphovascular invasion, perineural invasion, tumor grade, nodal status, tumor stage, and post-surgical treatment as covariates. In our multivariate analysis the model included *TP53* and *PIK3CA* mutational status, age, tumor focality, surgical margins, tumor depth, tumor grade, and nodal status.

The Kaplan-Meier analyses for OS and PFS compared four molecular conditions: *TP53* and *PIK3CA* mutated, *TP53* mutated only, *PIK3CA* mutated only, no *TP53* or *PIK3CA* mutation.

Results

Study cohort and pathology review

A total of 75 tissue samples, from 40 patients were included for analysis. Histology review of the cohort confirmed the presence of 33 VSCC, 8 VC, 13 dVIN, 11 ?dVIN, 6 DE-VIL, 2 VAAD, and 2 uVIN/HSIL samples (Fig. 1). Two cases originally diagnosed as well-differentiated VSCC were revised to VC after H&E review. One case comprised of areas of mixed VC and VSCC, both components were sampled and sequenced separately. Two cases had a prior history of possible VC. One case appeared to represent VAAD but there was moderate nuclear atypia worrisome for dVIN, therefore it was categorized as ?dVIN.

In 29 patients, at least one sample from both the invasive and in situ components could be matched from a single case. We also included 7 VSCC and 4 VC where the corresponding invasive or in situ samples could not be sequenced. In two cases (1 VSCC and 1 VAAD), the corresponding lesion (1?dVIN and 1 VSCC respectively) failed to be sequenced because the core missed the lesion on post-core H&E review. p16 IHC testing was negative in all but three samples, where morphologic features were also consistent with an HPV-associated neoplasm (two uVIN/HSIL and one VSCC). In one of these uVIN/HSIL cases, the associated VSCC was actually negative for p16 and the VSCC showed morphologic features of HPV-independence, such as keratinization and infiltrative growth, suggesting that the uVIN/HSIL in this case was an incidental/unrelated finding.

Mutational analysis

The mutational findings are summarized in Fig. 2 and Table 1.

HPV-independent (p16-negative) squamous lesions

Mutational analysis was successfully performed on 69 HPV-independent squamous lesions, including 25 cases with matched invasive and in situ samples. The NGS analysis identified significant mutations in 6 different genes: *TP53* ($n = 40$), *PIK3CA* ($n = 20$), *HRAS* ($n = 12$), *MET* ($n = 5$), *PTEN* ($n = 4$), and *BRAF* ($n = 1$). As shown in Fig. 2, there appeared to be two major molecular groups, one group with *TP53* mutations and a group without *TP53* mutations.

The first major group of lesions exhibited *TP53* mutations (61% [23/38] of patients; 58% of samples), with co-occurring mutations in *PIK3CA* (17%), *HRAS* (13%), *PTEN* (4%) and *MET* (4%). *TP53* was the most frequent mutation overall, occurring in 73% (22/30) VSCC, 85% (11/13) dVIN, and 70% (7/10) ?dVIN. No *TP53* mutations were

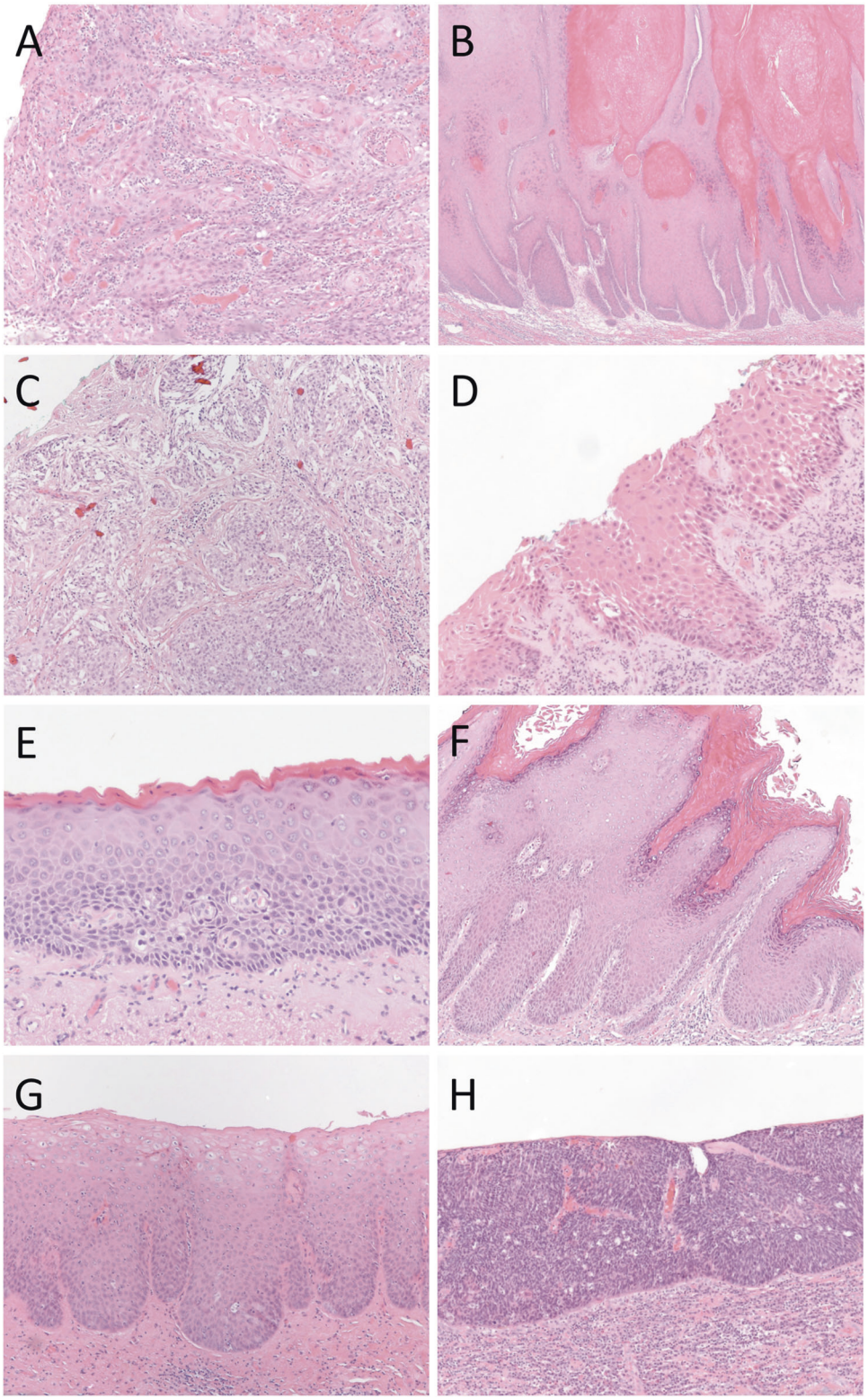

Fig. 1 Representative H&E images of vulvar squamous lesions included in the study. **a** HPV-independent VSCC, **b** HPV-independent VC, **c** HPV-associated VSCC, **d** dVIN, **e** ?dVIN, **f** DE-VIL, **g** VAAD, and **h** uVIN/HSIL.

found in VC (0/8), DE-VIL (0/6), or VAAD (0/2). Thus, evidence of *TP53* mutation was substantially more frequent in dVIN and ?dVIN compared to DE-VIL and VAAD ($p < 0.0001$). Similarly, invasive squamous carcinomas arising in association from dVIN and ?dVIN lesions were much more likely to be driven by *TP53* compared to other precursors ($p < 0.0001$). The presence of lichen sclerosus was not associated with *TP53* mutation ($p = 0.7676$).

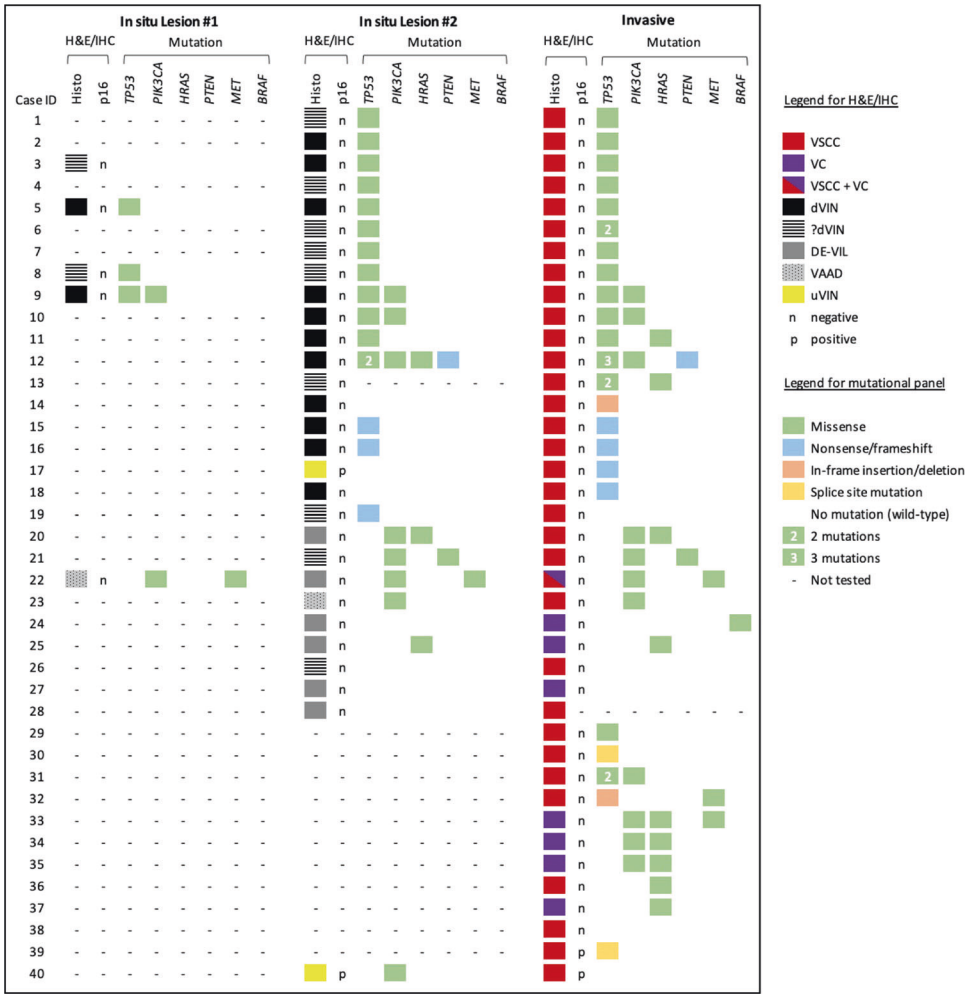

Fig. 2 Summary of the molecular findings in our cohort of invasive and in situ vulvar squamous lesions. The mutational status of all samples from 40 different patients is broken down in three panels, starting with precursor lesions on the left and center and the invasive tumors on the right.

Table 1 Rate of *TP53*, *PIK3CA*, and *HRAS* mutations within the different lesions.

Morphology	Type of mutations (%)		
	TP53	*PIK3CA*	*HRAS*
VSCC	22/30 (73)	8/30 (27)	4/30 (13)
VC	0/8 (0)	4/8 (50)	5/8 (63)
dVIN	11/13 (85)	4/13 (31)	1/13 (8)
?dVIN	7/10 (70)	0/10 (0)	0/10 (0)
DE-VIL	0/6 (0)	2/6 (33)	2/6 (33)
VAAD	0/2 (0)	2/2 (100)	0/2 (0)

VSCC vulvar squamous cell carcinoma, *VC* verrucous carcinomas, *dVIN* differentiated vulvar intraepithelial lesion, *?dVIN* suspicious for dVIN, *DE-VIL* differentiated exophytic vulvar intraepithelial lesion, *VAAD* vulvar acanthosis with altered.

Multiple *TP53* mutations were found in one dVIN (two missense mutations) and four VSCC (one case had two missense mutations, one case had three missense mutations, one case a missense and splice site mutation, one case had a missense and frameshift mutation).

The second group without *TP53* mutations (37% [15/38] of patients), was more molecularly heterogeneous. In this group, *PIK3CA* and *HRAS* mutations were both frequent; *PIK3CA* mutations were found in 29% (11/38) of patients (29% of samples) and *HRAS* mutations in 26% (10/38) patients (17% of samples). *HRAS* and *PIK3CA* mutations co-occurred in five patients (13%). *PIK3CA* and *HRAS* mutations were more frequent in cases without evidence of *TP53* mutations ($n = 7$ for both) than in lesions with *TP53* mutations ($n = 4$ and 3, respectively). All (100%) of the VC, DE-VIL and VAAD lesions resided within this group, compared to only 8/30 (27%) VSCC, 2/13 (15%) dVIN and 3/10 (30%) ?dVIN. VC, DE-VIL, VAAD and associated VSCC were more commonly mutated for *PIK3CA*, *HRAS*, *MET*, and *PTEN* compared to dVIN and ?dVIN and associated VSCC ($p = 0.0001$). One case showed morphologic features of both VSCC as well as VC and mutational analysis of both components were mutated for *PIK3CA* and *MET*, just like its associated DE-VIL. One VC had a *BRAF* mutation.

Two VSCC arising in association with ?dVIN, one unpaired VSCC, one VC, two dVIN, and three DE-VIL

(13% [9/69] of all samples), had no mutations detected by the targeted NGS panel. *PTEN* mutations were not specific to any molecular group or any particular histologic diagnosis.

In HPV-associated (p16-positive) squamous lesions

The representation of HPV-associated squamous lesions in our cohort was very small with only four samples, including one uVIN/HSIL associated with an VSCC, one VSCC, and one uVIN/HSIL that was an incidental finding adjacent to a p16-negative VSCC. A *PIK3CA* mutation was identified in one uVIN/HSIL and a *TP53* mutation (splice site mutation) was found in one unrelated VSCC.

Molecular progression

In 26 (25 p16-negative, 1 p16-positive) patients the invasive lesion was paired to at least one precursor lesion. Overall, 7/26 (27%) cases showed evidence of molecular progression. The invasive component often showed additional mutations (five gained *TP53*, one gained *HRAS*, one gained *BRAF* mutations) and in one case a different ($n = 1$, *TP53* c.817 C > G to c.817 C > T) mutation compared to the in situ component. Three cases had mutations in the in situ lesion (one *TP53*, one *HRAS*, one *PIK3CA*) that were not found in the paired VSCC.

Morphologic spectrum of dVIN lesions

We included 11 ?dVIN samples that had some but not all features of dVIN, all of which were adjacent to a VSCC. Morphologically, we identified two scenarios: (i) inflamed atrophic (or normal thickness) skin with mild to moderate basal atypia ($n = 8$ but only seven successfully cored), where the nuclear atypia was difficult to assess due to the confounding inflammation, and (ii) acanthotic lesions with mild (bordering on moderate) basal atypia ($n = 3$) (Fig. 3). While all cases with inflamed non-acanthotic skin/mucosa showed evidence of a *TP53* mutation, no *TP53* mutations were identified in the other group. Of those three cases with prominent acanthosis, one had an activating PIK3CA (c.3140 A > T) and *PTEN* (c.389 G > C) mutation, but no mutations were detected in the other two. Although this lesion was speculated to be dVIN, its mutational profile fits better under DE-VIL.

There were two cases called dVIN that did not harbor *TP53* mutations, although *TP53* mutations (one with a missense mutation, and the other with an in-frame deletion) were found in the adjacent VSCCs. On re-review both cases were still regarded as morphologically consistent with dVIN.

Survival analysis based on molecular characteristics

In a univariate analysis of the HPV-independent cases ($n = 36$), tumor depth of invasion ($p = 0.0430$) and perineural invasion ($p = 0.0025$) correlated with OS. The presence of a *TP53* status alone trended towards worse outcome but did not reach statistical significance ($p = 0.2160$). Kaplan-Meier analysis of *TP53* and *PIK3CA* mutations significantly showed that the worse OS was in tumors mutated for both *TP53* and *PIK3CA* versus no *TP53* or *PIK3CA* mutations ($p < 0.001$) (Fig. 4). Amongst the *TP53* mutated tumors, those with concurrent *PIK3CA* mutations had worse OS and PFS than those without *PIK3CA* mutations ($p = 0.0017$ and $p = 0.0337$ respectively).

The multivariate analysis also showed that the combination of *TP53* and *PIK3CA* mutations had a worse PFS compared to *TP53* or *PIK3CA* mutations alone, or neither mutations ($p = 0.0184$). *TP53* mutation status alone was not an independent predictor of OS or PFS. The other significant variables in our multivariate analysis were margin status (OS and PFS, $p = 0.0067$ and 0.0230), tumor focality (PFS, $p = 0.0184$), and nodal status (PFS, $p = 0.0009$).

Discussion

In this study we assessed the molecular profile of invasive and in situ squamous lesions of the vulva using targeted sequencing and correlated it with clinicopathologic features. Our analysis showed that, among HPV-independent cases, *TP53* mutations were present in almost all dVIN, ?dVIN, and associated VSCC, and absent in VC, DE-VIL, and VAAD, which were enriched in *PIK3CA* and *HRAS* mutations. dVIN was morphologically heterogenous, with basal atypia being the only consistent histologic feature. Finally, tumors with both *TP53* and *PIK3CA* had the worse survival profile.

The presence of *TP53* mutations in HPV-independent VSCC has ranged widely, between 17 and 44% using single-strand conformation polymorphism testing [32–34] and higher rates, 56–77%, with more recent Sanger and NGS technologies [35–39]. In our series, 76% of HPV-independent VSCC harbored *TP53* mutations, although this rate would probably be higher if we included a consecutive population-based series, because we had selectively enriched for VC, DE-VIL, and VAAD, entities which did not harbor *TP53* mutations. The majority of studies addressing the prognostic relevance of p53 in VSCC have largely used immunohistochemistry, with highly varied approaches to interpretation, leading to muddled results, as previously discussed by our group [28]. In the few studies that have evaluated *TP53* mutation status as a prognosticator, Nooij

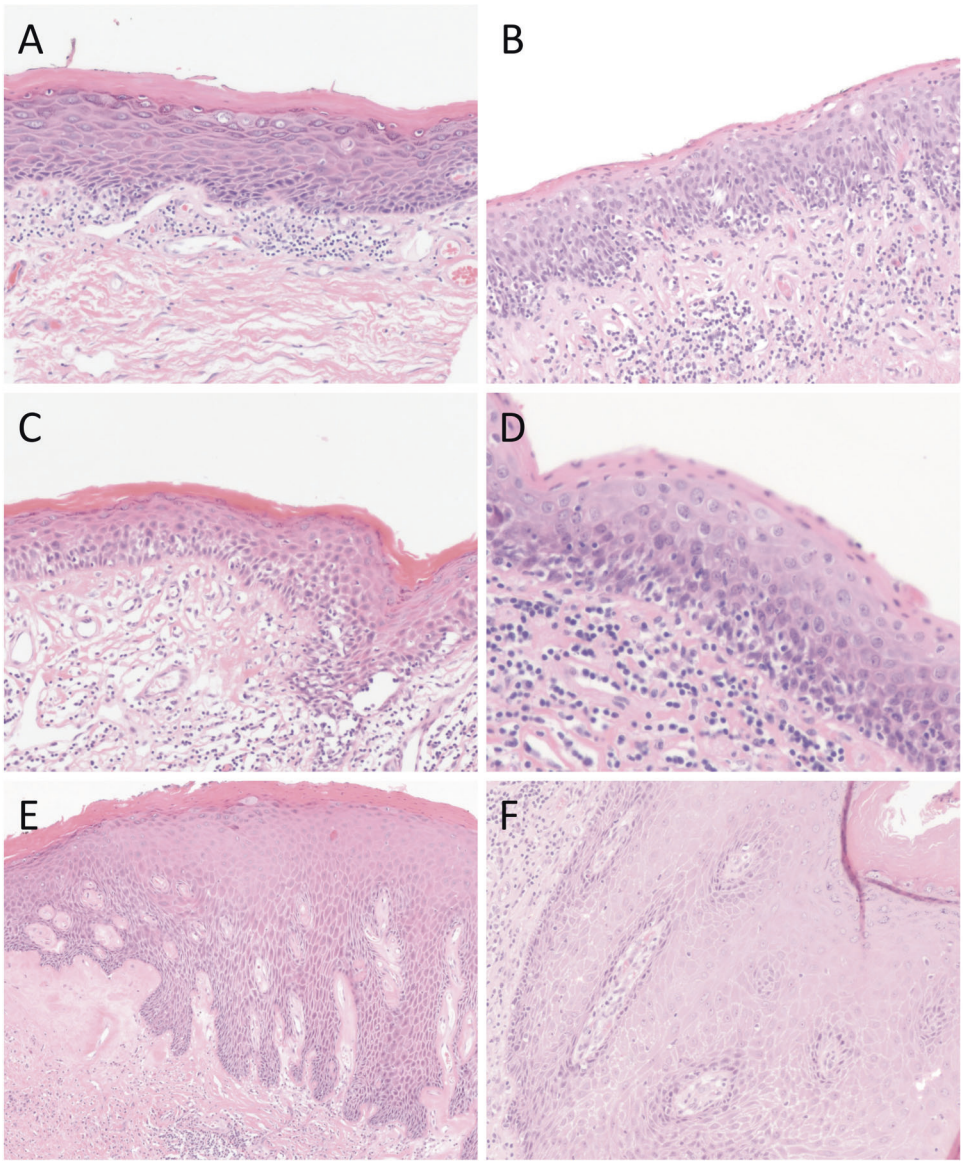

Fig. 3 H&E images of different ?dVIN lesions from our series, all of which had evidence of a *TP53* mutation. This includes (**a**–**d**) lesions with lesions with inflamed atrophic (or near normal thickness) skin with mild to moderate basal atypia, and (**e**, **f**) predominantly acanthotic lesion with mild basal atypia.

et al. have examined the largest series to date ($n = 118$) [38]. The authors found worse recurrence-free ($p = 0.044$) and disease-specific survival ($p = 0.049$) when comparing 3 groups of VC (HPV+, HPV−/p53 abn, HPV-/p53wt). However, when comparing the two groups directly, HPV-/p53abn vs HPV-/p53wt, no clinical or tumor characteristics were statistically different [38]. Kashofer et al. ($n = 72$) [37, 38] observed worse OS (69% vs 100% at 5 years) and Regauer et al. ($n = 24$) [40] noticed shorter disease-free intervals (33 vs 65 months) in *TP53* mutated tumors compared to *TP53* wild tumors, although no formal statistical analyses were made. In contrast, Trietch et al. ($n = 107$) and Choschzick et al. ($n = 25$) did not find that *TP53* was an independent prognosticator [35, 39]. In our small study, we found that *TP53* mutations alone were not an independent predictor of outcome, however, the combination of a *TP53* and *PIK3CA* mutations was a strong predictor of worse outcome. A much more sizeable series will be needed to clarify the prognostic significance of the molecular profile of vulvar squamous lesions.

TP53 was important in tumor progression, where 5/26 (19%) tumors showed additional *TP53* mutations in the invasive component compared to the adjacent in situ lesion. This acquisition of *TP53* mutations in tumor progression (in situ vs invasive lesions, primary vs recurrent VSCC) has been reported by other groups [37, 40–42]. Regauer et al. reported that 60% of patients with wild-type *TP53* in the primary VSCC, had a recurrence harboring a *TP53* mutation [40]. It is likely that the development multiple structural variations, associated with p53 dysfunction, over time increases malignant potential, but the details surrounding this process are still unknown [43, 44].

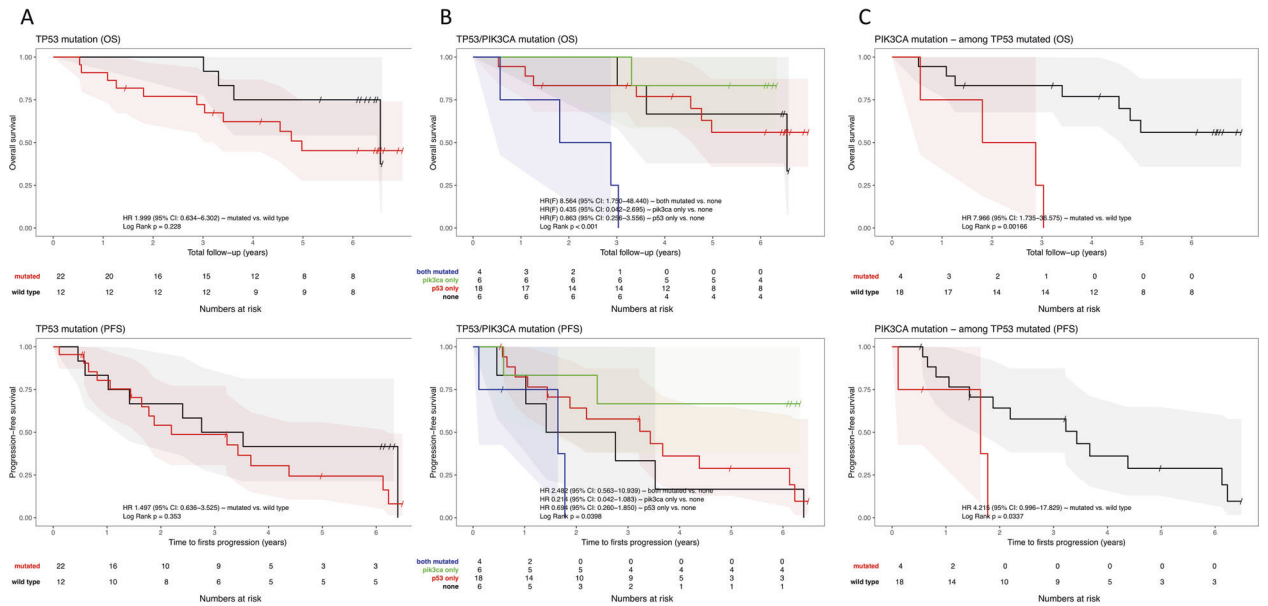

Fig. 4 Kaplan–Meier OS and PFS analyses in HPV-independent SCC. a *TP53* mutation versus no *TP53* mutation in all cases, **b** different combinations of *TP53* and *PIK3CA* mutational status in all cases, and **c** *PIK3CA* mutation among cases with *TP53* mutations.

In our cohort, the dVIN lesions, notorious for their rapid progression to invasive cancer, almost always showed identical mutations to their invasive counterpart and almost all harbored a *TP53* mutation [45]. This suggests that *TP53* is an early genetic event in the development of HPV-independent VSCC. Similar to Pinto et al., we also found multiple *TP53* mutations in dVIN. Pinto et al. further sequenced multiple foci of dVIN from four patients, and identified *TP53* mutations which overlapped amongst samples [46]. No two areas of dVIN were genetically distinct. The authors suggest that there may be multiple independent foci of in situ disease originating from different neoplastic clones. This concept of "field effect", akin to the phenomenon seen in colon cancers arising from inflammatory bowel disease and bladder cancer in smokers, makes intuitive sense given the frequent VSCC association with chronic vulvar inflammation. However, unless genetically distinct (non-overlapping) foci have been identified, we cannot confirm this theory, and it is possible that the mutations occur sequentially in tumors with high mutation burden, with the first being the main driver mutation and the second being a passenger.

The term dVIN was introduced by the International Society for the Study of Vulvovaginal Disease in 1986, based on the description of the entity by Abell in 1961, and is now a well-recognized morphologic entity in vulvar pathology [47, 48]. Its diagnosis however is challenging, especially at the lower end of the morphologic atypia spectrum [49]. There is tremendous variation across pathologists on how to approach the diagnosis of these lesions and what, if any, ancillary test should be used [50, 51]. Previous research has shown much higher proportions of dVIN without *TP53* alterations, reflecting its diagnostic irreproducibility. Our study showed that dVIN has a wide range of morphologies, and that current diagnostic criteria can lead to missing a large proportion of precursor lesions with clonal association to VSCC. The results from our ?dVIN group suggest that the diagnosis of dVIN should still be considered even in the absence of acanthosis, parakeratosis, hyperkeratosis, and even with moderate/marked atypia as the only finding. The presence of basal atypia was the only sensitive morphologic feature for dVIN, as previously suggested by others [52]. in situ lesions with prominent acanthosis, parakeratosis and hyperkeratosis without nuclear atypia often harbored *PIK3CA* and *HRAS* mutations, and are better classified under DE-VIL/VAAD. We also noted that the inflammatory reaction surrounding the squamous lesions often complicated the assessment of the basal atypia, especially in cases with an atrophic/non-acanthotic appearance. Pathologists should not confidently exclude the diagnosis of dVIN in a background of inflammation, in the presence of atypia.

The entities VAAD and DE-VIL can be enigmatic to the practicing pathologist. Although the concept of VAAD was published over 15 years ago, only 4 studies have since been published on this topic to date [38, 41, 53, 54]. Despite small numbers in our series, DE-VIL and VAAD were not associated with *TP53* mutations, instead they showed *PIK3CA* and/or *HRAS* mutations or sometimes no mutations. This is unlike what was shown in a previous small series of VAAD where all 7 VAAD lesions lacked *PIK3CA* mutations; Nooij et al. thereby proposed that *PIK3CA* status

may be used to differentiate VAAD from DE-VIL [38], because *PIK3CA* mutations were reported in >60% of DE-VIL by Watkins et al. [23]. However, 5/7 (71%) of VAAD by Nooij et al. did harbor *HRAS* mutations [38], a mutation that is also frequently found in DE-VIL (22–38%) [23, 55]. Certainly, there is significant morphologic and molecular overlap between DE-VIL and VAAD. The authors who initially described VAAD [24], in a subsequent study [23], combined VAAD, atypical verruciform hyperplasia, verruciform lichen simplex chronicus and verruciform dVIN, under an umbrella term "atypical verruciform lesions", and ultimately proposed a new name DE-VIL, which would encompass this spectrum of lesions. More importantly, the clinical outcomes for these p53 wild-type in situ lesions has not been reported. If the clinical differences between p53 mutated VIN (dVIN) and p53 wild-type lesions (DE-VIL, VAAD) can be confirmed, it seems prudent to adopt simpler nomenclature, such as p53-abn VIN and p53-wt VIN, as has been suggested avoiding many issues with overlapping morphologic features [26].

We recommend the use of p53 IHC or *TP53* sequencing in any HPV-independent lesions suspicious for dVIN, particularly when the lesion is close to a resection margin. Superimposed inflammation and normal thickness epithelium should not dissuade the pathologist. In our center, HPV status is first determined using p16 IHC. If the lesion is p16 negative and shows evidence of a p53 mutational staining pattern, dVIN can be diagnosed. The abnormal p53 pattern should match that seen in the adjacent squamous cell carcinoma. Comparison to wild-type p53 staining in hair follicles or normal skin is helpful. If there is no evidence of a p53 mutational pattern in the in situ lesion in question and the adjacent VSCC shows a p53 mutational pattern, then the lesion is likely reactive. If there is a question about DE-VIL or VAAD, p53 IHC will not help. The presence of *HRAS* or *PIK3CA* mutations support the diagnosis or DE-VIL/VAAD, but we understand most institutions will not have ready access to sequencing technologies. Currently, no reliable immunohistochemical biomarker for DE-VIL/VAAD exists. Lesions which raise the possibility of DE-VIL or VAAD, warrants close clinical follow-up.

Our NGS panel offers possibilities for targeted molecular therapies in VSCC. *PIK3CA*, *PTEN* and *HRAS* mutations may be amendable to mTOR and MEK inhibitors. We also identified *MET* and *BRAF* mutations, which may be susceptible to *MET* and *BRAF* inhibitors. However, we acknowledge that the efficacies of some of these novel therapies can be quite variable [56].

There are several limitations to our study. First the targeted mutation panel we used did not include certain genes that have been previously reported in squamous lesions of the vulva such as *CDKN2A*, *NOTCH1* or *ARID1A, BRCA2*, or *FBXW7* [23, 36, 38, 39]. As discussed above our NGS assay did not cover the entire *TP53* gene, and could have missed uncommon *TP53* alterations and large deletions [30]. Our sampling of HPV-associated lesions only included two uVIN/HSIL and two VSCC, which limits our ability to detect molecular overlap between HPV-associated and HPV-independent vulvar squamous lesions.

The diagnosis and stratification of HPV-independent vulvar squamous lesions are one of the most challenging areas in anatomical pathology, morphologic features are subtle and outcome data is rare at best. p53 IHC or *TP53* sequencing should be used to support the diagnosis of HPV-independent squamous lesions with basal atypia suggestive of dVIN. Our results support that *TP53* and *PIK3CA* mutation status can help to inform prognostication. We hope this study can lay the ground for more rigorous clinical and molecular studies to address the large gap in our knowledge, in this under-studied yet clinically important disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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