Human Pathology (2019) **92**, 67–80

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Human PATHOLOGY

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Original contribution

Recurrent genetic alterations and biomarker expression in primary and metastatic squamous cell carcinomas of the vulva☆,☆☆

Deyin Xing MD, PhD[a,b,*], Yuehua Liu MD[a], Hyeon Jin Park MS[c], Inji Baek MS[c], Hung Tran PhD[c], Gloria Cheang BS[c], Jorge Novo MD[a], Jessica Dillon MD[a], Andres Matoso MD[a], Emily Farmer BS[a], Max A. Cheng MS[a], Ya-Chea Tsai MS[a], Kara Lombardo MS[a], Michael G. Conner MD[d], Russell Vang MD[a,e], Chien-Fu Hung PhD[a,b], Tzyy-Choou Wu MD, PhD[a,b,e], Wei Song MD, PhD[c,]**

[a]*Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore 21231, MD*
[b]*Department of Oncology, The Johns Hopkins Medical Institutions, Baltimore 21231, MD*
[c]*Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York 10065, NY*
[d]*Department of Pathology, The University of Alabama at Birmingham, Birmingham 35233, AL*
[e]*Department of Gynecology and Obstetrics, The Johns Hopkins Medical Institutions, Baltimore 21231, MD*

Received 18 June 2019; revised 30 July 2019; accepted 7 August 2019

Keywords:
Mutations;
PD-L1;
Squamous cell carcinoma;
TP53;
Vulva

Summary Using a comprehensive next-generation sequencing pipeline (143 genes), Oncomine Comprehensive v.2, we analyzed genetic alterations on a set of vulvar squamous cell carcinomas (SCCs) with emphasis on the primary and metastatic samples from the same patient, to identify amenable therapeutic targets. Clinicopathologic features were reported and genomic DNA was extracted from 42 paraffin-embedded tumor tissues of 32 cases. PD-L1 expression was evaluated in 20 tumor tissues (10 cases with paired primary and metastatic tumors). Fifteen (88%) of 17 successfully analyzed HPV-unrelated SCCs harbored *TP53* mutations. 2 different *TP53* mutations had been detected in the same tumor in 4 of 15 cases. Other recurrent genetic alterations in this group of tumors included *CDKN2a* mutations (41%), *HRAS* mutations (12%), *NOTCH1* mutations (12%) and *BIRC3* (11q22.1–22.2) amplification (12%). Six HPV-related tumors harbored *PIK3CA, BAP1*, *PTEN*, *KDR*, *CTNNB1*, and *BRCA2* mutations, of which, one case also contained *TP53* mutation. Six cases showed identical mutations in paired primary site and distant metastatic location and four cases displayed different mutational profiles. PD-L1 expression was seen in 6 of 10 primary tumors and all 6 paired cases showed discordant PD-L1 expression in the primary and metastatic sites. Our results further confirmed the genetic alterations that are amenable to targeted therapy, offering the potential for individualized management strategies for the treatment of these aggressive tumors with different etiology. Discordant PD-L1 expression in the primary and metastatic vulvar SCCs highlights the importance of evaluation of PD-L1 expression in different locations to avoid false negative information provided for immunotherapy.
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☆ **Competing interest:** The authors have no competing interests to declare.

☆☆ **Grant Supports:** The present study was supported by the Career Enhancement Award by the Cervical Cancer SPORE program (P50CA098252) at Johns Hopkins (D.X.). This work was presented in part at the 2019 Annual Meeting of the United States and Canadian Academy of Pathology, National Harbor, MD.

* Correspondence to: Deyin Xing, Department of Pathology, The Johns Hopkins Hospital, Weinberg 2242, 401 N. Broadway, Baltimore, MD 21231.
** Correspondence to: Wei Song, Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, 1300 York Avenue, LC-904, New York, NY 10065.
E-mail addresses: dxing2@jhmi.edu (D. Xing), sow2005@med.cornell.edu (W. Song).

https://doi.org/10.1016/j.humpath.2019.08.003
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1. Introduction

It has been estimated that approximately 6070 new cases of vulvar cancer will be diagnosed in the US in 2019, with roughly 1280 deaths, accounting for 4–5% of all gynecologic cancers [1]. Over 90% of malignant neoplasms of the vulva are squamous cell carcinomas (SCCs). While nearly all SCCs of the uterine cervix are caused by carcinogenic types of high-risk human papillomavirus (HPV), SCCs of the vulva may or may not be associated with high-risk HPV infection [2]. In fact, similar to uterine cervix, HPV-related vulvar SCC often originates from usual type high-grade squamous intraepithelial lesions (HSIL) and usually presents in younger women. In contrast, HPV-unrelated carcinoma is associated with differentiated vulvar intraepithelial neoplasia (dVIN) and/or lichen sclerosus that mainly involves older women [3-5].

Etiologically, HPV-unrelated SCCs of the vulva usually harbor *TP53* somatic mutations that are also detected in the precursor lesions dVIN and lichen sclerosus [6-8]. While studies on somatic mutations other than those in the *TP53* gene used to be hampered by the absence of analytical tools, recently developed next-generation sequencing (NGS) technology allows for analysis of mutations and other genetic alterations, such as copy number change and translocations, in multiple genes. In addition, the potential application of molecule/pathway-based targeted therapies to vulvar SCC largely relies on identification of genetic alterations that are amenable to such therapies in these tumors. Using this technology, one study analyzed 43 cases of vulvar SCC and found high mutation rates in both HPV-positive and HPV-negative disease with the former characterized by oncogenic mutations in *PIK3CA* (27%), *FGFR3* (14%), and *PTEN* (9%) and the latter characterized by mutations in *TP53* (57%), *HRAS* (24%), *PIK3CA* (19%), and *CDKN2a* (14%) [9]. Another study employed targeted NGS that covered 47 genes and identified recurrent hotspot mutations in *TP53*, *PIK3CA*, *BRCA2*, *HRAS*, *FBXW7*, *ERBB4*, and *GNAS* [10]. It is of interest that a screen study for hotspot mutations in 50 genes revealed similar mutational rates of *TP53* and *CDKN2a* in HPV-related and HPV-unrelated SCC of the vulva, suggesting that, despite originating from different precursor lesions, oncogenic mechanisms involving two pathways of vulvar SCC histopathogenesis may be similar [11].

It has been reported that HPV-unrelated SCCs of the vulva tend to have an unfavorable prognosis compared to HPV-related vulvar SCCs [3]. In either situation, lymph node status is considered the most important independent factor that is related to patient outcome [12-14]. The 5-year survival rate for patients with distant metastasis is less than 20%. However, the underlying mechanisms by which these tumors metastasize to distant locations, especially lymph nodes, remains largely unknown. One of above studies compared protein and genomic alterations in SCCs of the vulva by primary *versus* metastatic site [10]. The study demonstrated that metastatic tumor samples have a higher rate of TLE3, but lower rate of PD-L1 expression. However, this study only assessed hotspot mutations in a limited panel of 47 genes. Moreover, the tissues from primary and metastatic sites used for immunohistochemistry and molecular profiling were attained from different patients, thus confounding the data interpretation. In this study, we analyzed genetic alterations on a set of vulvar SCCs with emphasis on the primary and metastatic samples from the same patient, to investigate the biology and genetics of tumor progression and identify amenable therapeutic targets.

2. Material and methods

2.1. Case selection

42 samples from 32 cases of vulvar SCCs were identified in the files of the Johns Hopkins Hospital from 2002 to 2017 (31 cases) and the University of Alabama at Birmingham (1 case). Of which, 10 cases had concurrent (9 cases) or subsequent (1 case) distant metastasis, including 9 cases to the lymph nodes and 1 case to the groin region. The specimens from the primary lesions in this series included 5 biopsies and 27 local or wide excisions. Histologic sections of these cases were re-reviewed by two pathologists (D.X. and W.S.) to verify the diagnosis and assess the tumor content and specimen quality for the further analysis. The study was approved by the Institutional Review Board at the Johns Hopkins Hospital.

2.2. Immunohistochemistry and in situ hybridization

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections as previously described [15]. Markers used included: p16 (INK4a) (mouse monoclonal, Ventana, Tucson, AZ; prediluted); p53 (mouse monoclonal, Ventana, Tucson, AZ; prediluted); PD-L1 (mouse monoclonal, 22C3, Dako/Agilent, Santa Clara, CA). In situ hybridization was performed using a high-risk HPV RNA probe solution (RNAscope, Advanced Cell Diagnostics, Newark, CA; HPV types16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82).

2.3. Semi-quantitative assessment of PD-L1 protein expression

PD-L1 protein expression was determined by Combined Positive Score (CPS) that were evaluated based on the number of PD-L1-positive cells (tumor, lymphocytes, and macrophages) in relation to total tumor cells [16]. CPS was calculated as follows:

$$\frac{\#\text{of PD–L1 staining cells (tumor cells + lymphocytes + macrophages)}}{\text{total\#of viable tumor cells}} \times 100.$$

Although the CPS can exceed 100, the maximum score is defined as CPS of 100. Interpretation of PD-L1 expression is as follows: CPS< 1, no PD-L1 expression; CPS of at least 1,

PD-L1 expression. CPS was assessed by four pathologists (D.X., J.N., J.D., A.M.) and an average score with standard deviation was calculated. Of note, evaluation of PD-L1 expression was a subjective assessment.

2.4. DNA extraction

Paraffin-embedded tumor tissues, identified by H&E staining of adjacent sections (tumor elements account for more than 70% of section area), were macrodissected, and genomic DNA was extracted using a QIAamp DNA FFPE Tissue Kit with an adapted protocol (Qiagen, Valencia, CA), as previously described. Briefly, slides bearing paraffin-embedded tissues were baked at 68 °C for 20 to 30 seconds; the tissues were deparaffinized 3 times with xylene, and residual xylene was removed by washing through serial dilutions of ethanol. The tumor tissues were separated from adjacent normal tissues and placed in a tube allowing for complete evaporation of residual ethanol. The tissue pellet was resuspended in Buffer ATL (Qiagen) with added proteinase K. The rest of the procedure followed the manufacturer's instruction.

2.5. Targeted next-generation sequencing and data analysis

The extracted DNA was amplified by the Oncomine Comprehensive Panel (OCP) v2 and subjected to NGS using the Ion Torrent S5™ system (Life Technologies) according to the vender recommendations. The Oncomine Comprehensive assay was developed and its performance characteristics were determined by the Clinical Genomics Laboratory, Department of Pathology and Laboratory Medicine at Weill Cornell Medicine/New York-Presbyterian Hospital [17] and approved by the New York-State Department of Health (NYS-DOH). The targeted gene panel interrogates 143 unique cancer genes including the hotspots of 73 genes, 49 copy number alteration (CNA) genes, entire coding regions of 26 genes, and 22 fusion driver genes (Supplementary Table 1). OCP is designed to detect all types of mutations: single nucleotide variants (SNVs), insertion/deletion (Indel), copy number variants (CNVs), and gene fusions. The data obtained were analyzed with the Ion Reporter™ Software 5.6 including Coverage Analysis and Torrent Variant Annotator v2.3 plug-ins. The mutation nomenclature is based on the recommendations from the Human Genome Variation Society (http://www.hgvs.org/mutnomen).

2.6. Statistical analysis

The unpaired t-test procedure compares means of the age for two groups of cases. Prognostic factors predictive of cause-specific survival (CSS) were analyzed using univariate hazard model, calculated using the Kaplan–Meier method and compared using the log-rank test. Statistical analyses were performed with SAS v 9.4 (SAS Institute, Cary, NC, USA). A value of $P < .05$ was considered to be statistically significant.

3. Results

3.1. Clinicopathologic features of vulvar SCCs

Clinicopathologic features are summarized in Table and Supplementary Table 2. A total of 20 cases (18 White, 2 African American) were HPV-unrelated and the patients with this type of tumor ranged in age from 25 to 92 (mean, 70; median, 74). Tumor size ranged from 0.25 to 14.0 cm (mean, 3.9; median, 3.0). Most of these tumors were well-differentiated SCCs with variable maturation with keratin pearls and immature keratinocytes. Occasionally, focal moderately differentiated areas can be seen. By report, dVIN and/or lichen sclerosus, putative precursor lesion of these tumors, was present in 11 of 20 cases. Precursor lesion information was obtained by report, slides not reviewed. Lymph node dissection was performed in 16 patients, of which, 10 cases were present with positive lymph nodes. Twelve patients with HPV-related SCCs of the vulva were included in this study, who ranged in age from 25 to 75 (mean, 50; median 51), a range significantly younger than that of HPV-unrelated SCCs of the vulva ($P = .001$, unpaired *t*-test). Tumor size was available in 9 cases, ranging from 0.2 to 5.0 cm (mean, 2.1 cm; median, 1.8 cm). In keeping with the previous description, these tumors were usually basaloid or warty type which may resemble high-grade squamous intraepithelial lesion (HSIL/VIN 2–3). The latter was the precursor lesion of HPV-related SCCs of the vulva and was present in 9 of 12 cases. Lymph node dissection was performed in 11 patients, of which, 4 cases had nodal metastasis.

3.2. Genetic alterations of HPV-unrelated SCCs of the vulva

Table and Fig. 1 summarized the successfully analyzed pathogenic variants in 36 of 42 samples (26 cases). Mutational profile and copy number variants (CNVs) analysis failed in 6 cases due to poor DNA quality (Supplementary Table 2). Detailed information about genetic alterations are also described in Supplementary Table 3. All 20 HPV-unrelated tumors displayed either negative or focal/patchy p16 staining pattern. To further confirm the HPV status, in situ hybridization analysis was performed in 4 selected tumors and none showed detectable HPVs. Mutational profile and CNVs were successfully analyzed in 17 cases (Table and Fig. 1A). It is of interest that 15 (88%) of 17 HPV-unrelated SCCs harbored TP53 mutations. 2 different *TP53* mutations had been detected in the same tumor in 4 of 15 cases. *CDKN2a* (*p16*) mutations were present in 7 cases (5 nonsense mutations and 2 missense mutations). One tumor

Table Clinicopathologic features and genetic alteration profile

Case	Age	Race	Procedure	Primary tumor location	Size (cm)	Depth of invasion (cm)	Precursor lesion [a]	P16	LN [b] dissection	LN [b] metastasis	Mutations	CNV [c]	Follow-up
1 d	76	White	Partial vulvectomy	Right vulva; left vulva; clitoris	4.8	1.3	dVIN	Positive (patchy)	Yes	Yes	*TP53* p.Arg282Trp; *CDKN2A* p.His83Tyr (primary) *TP53* p.Arg282Trp; *CDKN2A* p.His83Tyr (metastatic)	– –	25 mo, dead of disease
2 d	76	White	Radical Hemivulvectomy	Right vulva	5.0	2.2	–	Negative	Yes	Yes	*TP53* p.Arg175His; *CDKN2A* p.Trp110Ter (primary) *TP53* p.Arg175His; *CDKN2A* p.Trp110Ter (metastatic)	– –	24 mo, dead of disease
3 d	79	White	Radical Hemivulvectomy	Left vulva	5.5	Full thickness	–	Positive (patchy)	Yes	Yes	*TP53* p.Asn131del; *HRAS* p.Gly13Arg (primary) *TP53* p.Asn131del; *HRAS* p.Gly13Arg (metastatic)	– –	3.5 mo, dead of disease
4 d	66	White	Anterior exenteration	Vulva, vagina, urethra, bladder	3.6	Full thickness	–	Negative	Yes	Yes	*TP53* p.Arg342fs (primary) *TP53* p.Arg342fs (metastatic)	*BIRC3* *BIRC2* –	4.5 mo, dead of disease
5 d	58	White	Radical vulvectomy	Right vulva, near midline	2.1	0.35	–	Positive (patchy)	Yes	Yes	*TP53* p.Arg213Ter; *CDKN2A* p.Pro70Leu; *PTPN11* p.Thr468Met (primary) *TP53* p.Arg213Ter; *NF1* p.Trp696Ter (metastatic)	*MCL1*; *MYC*; *BIRC3*; *CDK4* *APEX1* –	13 mo, dead of disease
6 d	49	White	Radical partial vulvectomy	Right vulva, near clitoris	3.0	0.6	dVIN	Negative	Yes	Yes	*TP53* p.Arg196Ter (primary) - (metastatic)	– –	12 mo, no evidence of disease
7 d	25	White	Radical hemivulvectomy	Left vulva, clitoris	3.0	0.9	–	Negative	Yes	Yes	*TP53* p.Arg175His; *CDKN2A* p.Arg80Ter (primary) *TP53* p.Arg175His (metastatic)	– –	9 mo, no evidence of disease

8 d	46	African American	Biopsy	Left and right vulva	–	–	dVIN	Negative	No	No	*TP53* p.Gly266Glu; *TP53* p.Arg342Ter; *CDKN2A* p.Arg80Ter (primary)	–	13 mo, dead of disease
											TP53 p.Gly266Glu; *TP53* p.Arg342Ter; *CDKN2A* p.Arg80Ter (metastatic)	–	
9 d	72	White	Partial vulvectomy	Vulva	14	1.4	dVIN	Positive (patchy)	Yes	Yes	*TP53* p.Val143Met; *HRAS* p.Gly12Ser (primary)	–	11 mo, dead of disease
											TP53 p.Val143Met; *HRAS* p.Gly12Ser (metastatic)	–	
10	82	White	Radical local excision	Left vulva	1.8	0.5	–	Negative	Yes	Yes	*TP53* p.Gly245Ser; *TP53* p.Arg248Gln; *CDKN2A* p.Arg58Ter	–	41 mo, dead of disease
11	55	African American	Left hemivulvectomy	Left vulva	2.0	0.4	Lichen sclerosus	Positive (patchy)	Yes	No	*TP53* p.Ser94Ter; *TP53* p.His193Tyr; *NOTCH1* p.Ala465Thr	–	105 mo, dead of disease
12	64	White	Radical posterior vulvectomy	vulva	1.4	0.3	Lichen Sclerosus/ dVIN	Negative	Yes	No	*TP53* p.Ser90fs	–	36 mo, no evidence of disease
13	92	White	Radical vulvectomy	Left vulva	2.7	0.9	–	Positive (patchy)	Yes	No	*TP53* p.Gln165fs d16; *PIK3CA* p.Glu542Lys	Cyclin D1	2 mo, no evidence of disease
14	85	White	Radical vulvectomy	Vulva, clitoris	2.5	0.7	Lichen sclerosus	Positive (patchy)	Yes	No	*TP53* p.Arg273Cys; *TP53* p.Tyr327Ter; *CDKN2A* p.Arg80Ter; *NOTCH1* p.Gln154fs d123; *ERBB4* p.Glu452Ter	–	5 mo, no evidence of disease
15	84	White	Right vulvectomy	Right vulva	3.2	1.2	–	Positive (patchy)	Yes	No	*TP53* p.Arg249Met; *BAP1* p.Gly380fs	*CDK6*	35 mo, dead of disease
16	61	White	Local excision	Left vulva, labium	0.8	0.2	dVIN	Positive (patchy)	No	No	Not detected	–	30 mo, recurrent, alive
17	87	White	Excisional biopsy	Vulva	0.25	0.2	Lichen sclerosus/ dVIN	Positive (patchy)	No	No	Not detected	–	12 mo, dead of disease
18	44	White	Biopsy	Left vulva	–	–	–	Positive (diffuse)	No	No	*CTNNB1* p.Gln4Ter; *BRCA2* p.Leu2587Phe	–	Loss to follow-up
19 e	47	White	Partial radical vulvectomy	Left vulva	5.0	3.0	HSIL/VIN 3	Positive (diffuse)	Yes	Yes	*PIK3CA* p.Glu545Lys; *FGFR3* p.Ser249Cys (primary)	–	30 mo, no evidence of disease

(continued on next page)

Table (*continued.*)

Case	Age	Race	Procedure	Primary tumor location	Size (cm)	Depth of invasion (cm)	Precursor lesion [a]	P16	LN [b] dissection	LN [b] metastasis	Mutations	CNV [c]	Follow-up
												–	
											PIK3CA p.Glu545Lys (metastatic)		
20	70	White	Simple vulvectomy	Posterior vulva	0.2	0.2	HSIL/VIN 3	Positive (diffuse)	Yes	Yes	*BAP1* p.Ser460Ter *PTEN* Gly129Arg	*MYCL*	17 mo, no evidence of disease
21	54	African American	Radical vulvectomy and clitorectomy	Left vulva and clitoris	3.3	0.5	HSIL/VIN 3	Positive (diffuse)	Yes	No	*KDR* p.Ser1100Phe	–	45 mo, no evidence of disease
22	51	African American	Biopsy	Right posterior vulva	–	–	HSIL/VIN 3	Positive (diffuse)	Yes	No	*NFE2L2* p.Asp27Asn *PIK3CA* p.Glu545Lys	–	41 mo, no evidence of disease
23	50	African American	Local excision	Posterior vulva/vagina	–	0.75	HSIL/VIN3/ VAIN3	Positive (diffuse)	Yes	No	*TP53* p.Gly245Ser; *RB1* p.Gln770fs [d]40; *ATM* p.Gln2733Ter; *PIK3CA* p.Glu542Lys; *NF1* p.Trp696Ter	–	160 mo, no evidence of disease
24	25	African American	Local excision	Vulva	1.0	0.2	HSIL/VIN3	Positive (diffuse)	Yes	Yes	Not detected	–	83 mo, no evidence of disease
25	53	White	Posterior partial vulvectomy	Posterior vulva	4.5	1.1	–	Positive (diffuse)	Yes	Yes	Not detected	–	18 mo, no evidence of disease
26	49	White	Partial vulvectomy	Right vulva	1.8	0.2	HSIL/VIN3	Positive (diffuse)	Yes	No	Not detected	–	34 mo, no evidence of disease

Abbreviations: dVIN, differentiated-type vulvar intraepithelial neoplasia; HSIL/VIN 3/VAIN 3, High grade squamous intraepithelial lesion/vulvar intraepithelial neoplasia 3/vaginal intraepithelial neoplasia 3.

[a] Precursor lesion information is obtained by report, slides not.

[b] LN: lymph node.

[c] CNV: copy number variant.

[d] Paired primary and metastatic (HPV-unrelated).

[e] Paired primary and metastatic (HPV-related).

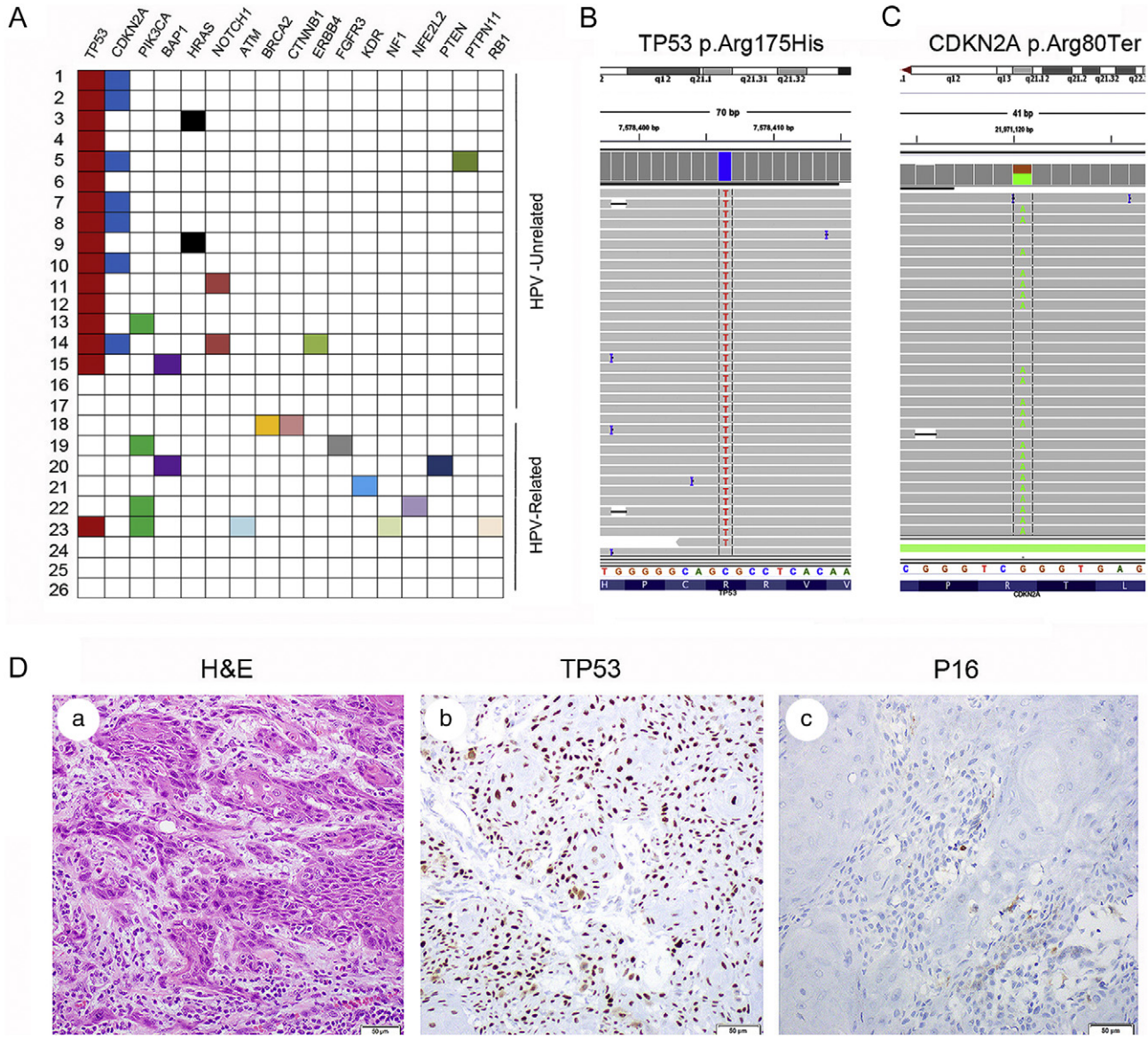

Figure 1 Somatic mutations detected by next-generation sequencing and a representative case with *TP53* and *CDKN2a* mutations. The mutated activating oncogenic and caretaker tumor suppressor genes in successfully sequenced vulvar SCCs are plotted (arranged in descending order of number of mutations, and if the same number then in an alphabetic order, A). A representative case (case no. 7) with mutations of *TP53* (p.Arg175His missense mutation, B) and *CDKN2a* (p.Arg80Ter nonsense, C) are shown in the middle and right. Consistent with genotype, the tumor (D-a, H&E) demonstrates aberrant/mutation-type p53 over-expression (D-b) consistent with a missense mutation staining pattern. The tumor also shows a negative p16 staining with positive internal control, consistent with *CDKN2a* truncation mutation pattern (D-c).

(case no. 7) from a 25 year-old patient contained *TP53* p.Arg175His missense mutation (Fig. 1B) that resulted in a strong and diffuse p53 expression pattern (Fig. 1D). The same tumor demonstrated a complete negative p16 immunostaining pattern that correlated with a *CDKN2a* truncation mutation genotype (Fig. 1C and D). An in situ hybridization analysis failed to detect high-risk HPVs in this patient's tumor. Another tumor (case no. 13) harbored a *TP53* insertion mutation that led to protein truncation at amino acid 16 after the insertion site. Consistently, the tumor demonstrated complete absence of p53 expression, consistent with the "null" pattern of aberrant/mutation-type p53 expression (Fig. 2A and B). This tumor also had focal p16 expression and contained *PIK3CA* somatic hotspot mutation (p.Glu542Lys) (Fig. 2A and C). Other recurrent somatic mutations in this group of tumors included *HRAS* (2 cases) and *NOTCH1* (2 cases). *BAP1* (p.Gly380fs), *ERBB4* (p.Glu452Ter), and *PTPN11* (p.Thr468Met) were detected individually in 3 tumors that also harbored other mutations. Consistent with a frame shift *BAP1* mutation that led to a non-functional protein, immunohistochemical staining revealed loss of BAP1 expression in case no. 15 (Supplementary Fig. S1). Two cases (case no. 4 and no. 5) showed copy number gains with *BIRC3* (11q22.1–22.2) amplification. *BIRC2*, another gene

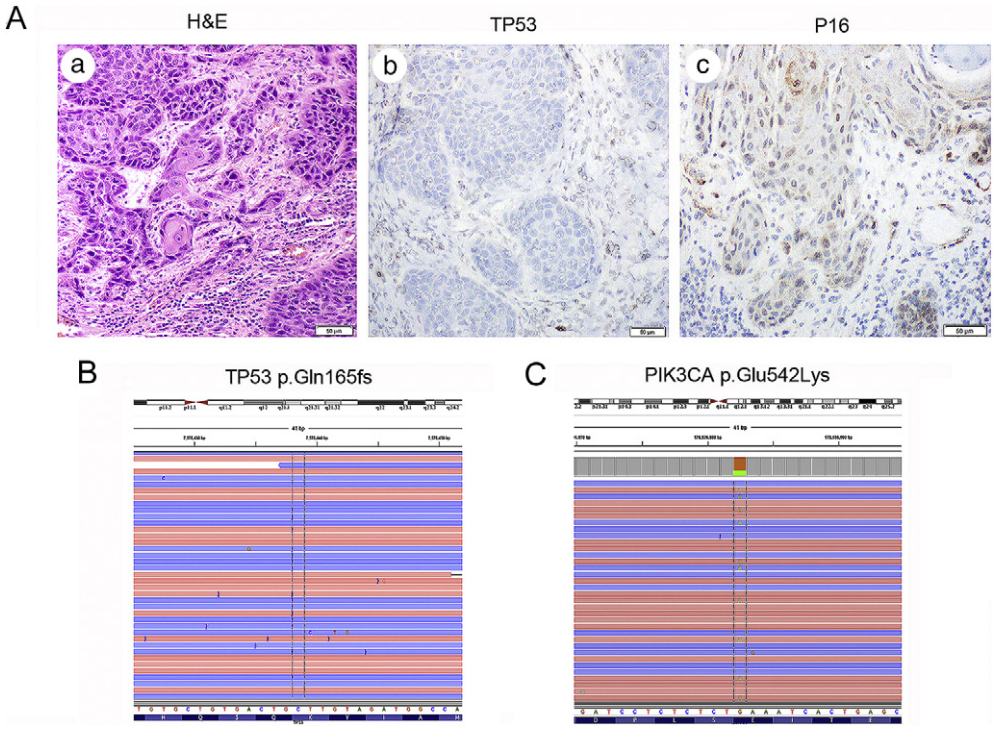

Figure 2 A representative HPV-unrelated SCC case (case no. 13) with mutations of *TP53* and *PIK3CA*. The tumor (A-a, H&E) demonstrates aberrant/mutation-type complete loss of p53 expression ("null" pattern, A-b) because truncated p53 protein (p.Gln165fs, B) cannot be recognized by the p53 antibody. The tumor demonstrates a focal/patchy p16 staining (A-c). *PIK3CA* (p.Glu542Lys) mutation is also detected (C).

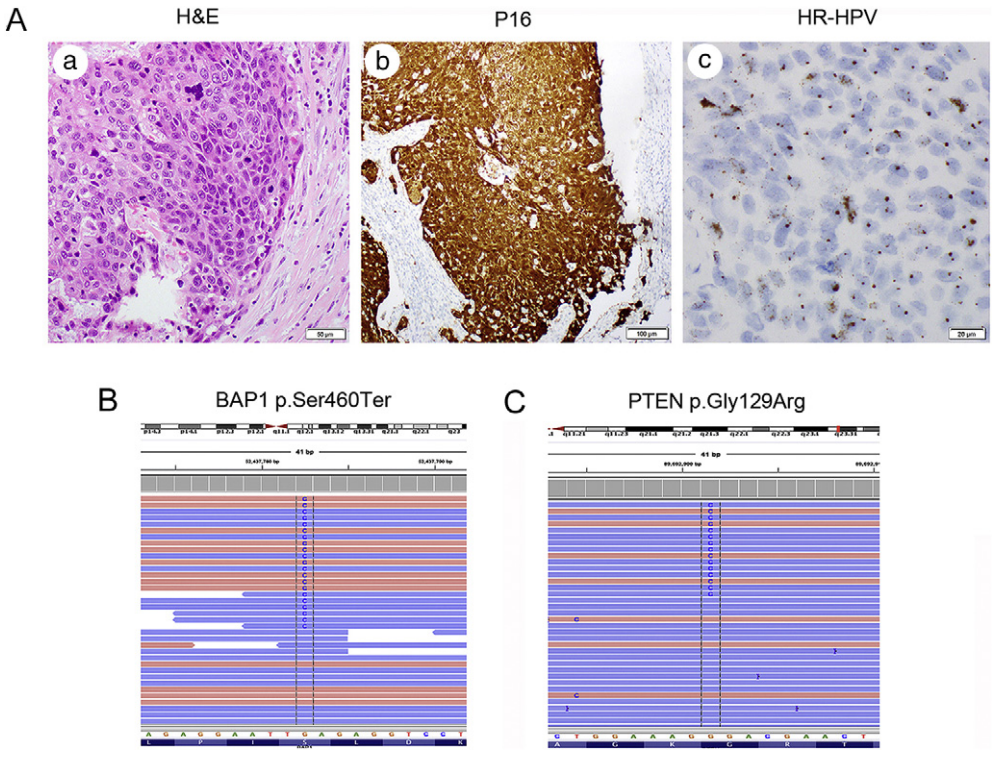

Figure 3 A representative HPV-related SCC case (case no. 20) with mutations of *BAP1* and *PTEN*. The tumor (A-a, H&E) demonstrates strong and diffuse p16 (A-b) with detected high-risk HPVs (A-c) by RNA in situ hybridization. The tumor harbors *BAP1* p.Ser460Ter nonsense mutation (B) and *PTEN* p.Gly129Arg missense mutation (C).

in the same locus, also displayed amplification in case 4. Amplifications of *MCL1*, *MYC*, *CDK4*, and *APEX1* were present in case no. 5. Amplification of *cyclin D1* was detected in case no. 13 and *CDK6* in case no. 15, respectively.

3.3. Genetic alterations of HPV-related SCCs of the vulva

All 12 HPV-related tumors displayed strong and diffuse p16 staining pattern. High-risk HPV in situ hybridization analysis was performed in 4 tumors, of which, 3 tumors demonstrated detectable high-risk HPVs. Mutational profile and CNVs were successfully analyzed in 9 cases, of which, 6 tumors contained somatic mutations that were included in this panel (Table). Three HPV-related tumors harbored *PIK3CA* hotspot mutations (2 with p.Glu545Lys and 1 with p.Glu542Lys), of which 1 tumor also contained *TP53* mutation. Other somatic mutations coexisted with *PIK3CA* included *FGFR3* (p.Ser249Cys), *NFE2L2* (p.Asp27Asn), *RB1* (p.Gln770fs), *ATM* (p.Gln2733Ter), and *NF1* (p.Trp696Ter). Three HPV-related tumors had no PIK3CA mutation: case no. 18 with *CTNNB1* (p.Gln4Ter) and *BRCA2* (p.Leu2587Phe) mutations; case no. 20

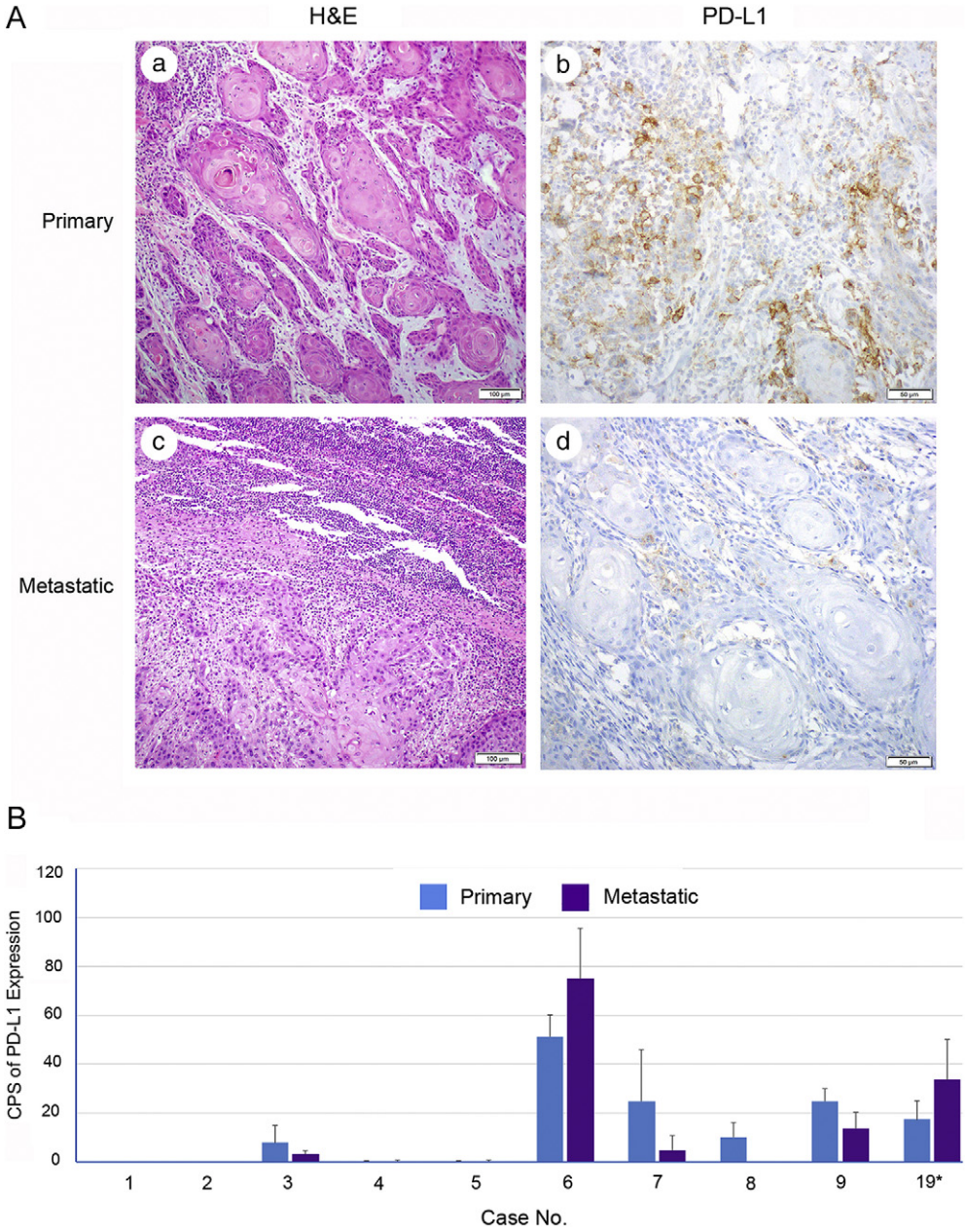

Figure 4 Semi-quantitative assessment of PD-L1 expression in paired primary and metastatic SCCs of the vulva. A representative primary vulvar SCC (case 7, A-a, H&E) shows PD-L1 expression with CPS approximately 23 (A-b); metastatic tumor (A-c, H&E) demonstrates decreased PD-L1 expression with CPS approximately 4 (A-d). A schematic graph of CPS of PD-L1 expression in 10 paired primary and metastatic SCCs of the vulva (B). * Case 19, HPV-related.

(strong and diffuse p16 staining and detected high-risk HPV, Fig. 3A) with *BAP1* (p.Ser460Ter) and *PTEN* (p.Gly129Arg) mutations (Fig. 3B and C); case no. 21 with *KDR* (p.Ser1100Phe) mutation. Copy number variation analysis revealed that *MYCL* amplification was detected in 1 tumor with *BAP1* and *PTEN* mutations (case no. 20).

3.4. Comparative genetic alterations of primary and metastatic SCCs of the vulva

Comparative mutational and copy number changes in primary and metastatic SCC of the vulva were successfully assessed in 10, paired primary and metastatic SCCs of the vulva and summarized in Table. Six cases showed identical mutations in primary site and distant metastatic location (5 cases in lymph nodes and 1 case in the groin region). It is of interest that 4 cases displayed different mutational profile: *CDKN2a* p.Pro70Leu and *PTPN11* p.Thr468Met mutations only present in the vulva, instead, *NF1* p.Trp696Ter only present in the lymph node (case no. 5); *TP53* p.Arg196Ter and *CDKN2a* p.Arg80Ter only present in the vulva but not in the inguinal lymph nodes (case no. 6 and case no. 7, respectively); *FGFR3* p.Ser249Cys mutation present in primary SCCs of the left vulva but not in left inguinofemoral lymph node (case no. 19). Likewise, gene amplifications were only identified in the primary rather than metastatic tumors (case no. 4 and case no. 5).

3.5. PD-L1 expression in primary and metastatic SCCs of the vulva

Ten primary-metastasis tumor pairs were tested for PD-L1 expression that was assessed by CPS. PD-L1 expression was seen in 6 primary tumors with CPS $\geq$ 1; 4 primary tumors with no PD-L1 expression (Fig. 4A and B). While case no. 19 was HPV-related, all others were HPV-unrelated. All 6 cases showed discordant PD-L1 expression: 2 showed increased PD-L1 expression in the metastasis; 4 showed a decrease in PD-L1 expression in the metastasis, with 1 from positive to negative (case no. 8, Fig. 4B).

3.6. Clinical outcome of patients with SCCs of the vulva

Follow-up information was available for 30 patients (Table and Supplementary Table 1). Nine patients with

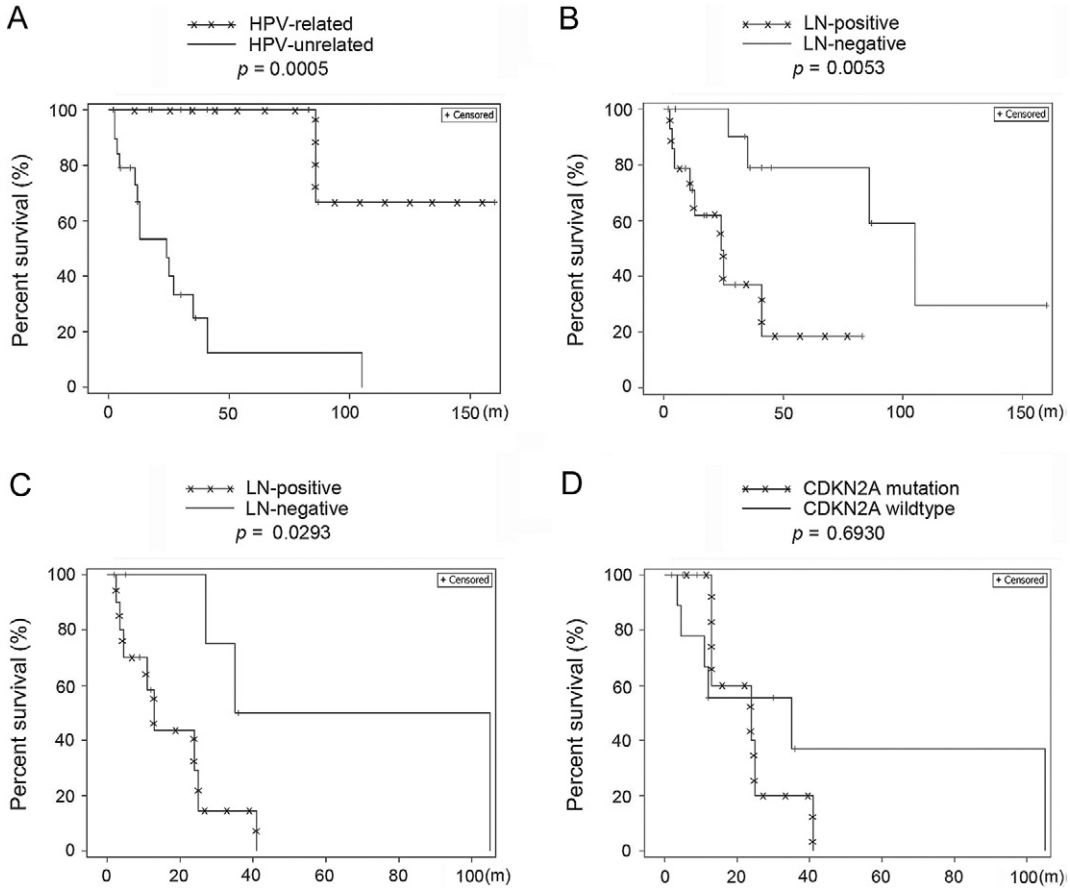

Figure 5 Factors related to cause specific survival (CSS) in patients with vulvar SCCs by a univariate analysis. HPV-related etiology demonstrates a superior CSS (A). Negative lymph node involvement shows a more favorable CSS in all SCCs of the vulva (B) and HPV-unrelated tumors (C). *CDKN2a* mutations was not associated with prognosis in the patients with HPV-unrelated SCCs of the vulva (D). LN: lymph node; m: month.

HPV-related SCCs of the vulva with available follow-up information in the current study were all alive at follow-up intervals ranging from 18 months to 160 months (median, 41 months). One patient (case no. 31) died of disease with a follow-up of 86 months. In contrast, 15 of 20 patients with HPV-unrelated SCCs died of disease at follow-up intervals ranging from 2.5 months to 105 months (median, 13 months). HPV infection was a significant prognostic factor for cause specific survival (CSS) by a univariate analysis (P = .0005, Fig. 5A). Lymph node metastatic status was significantly associated with CSS for all patients with SCC of the vulva, regardless of HPV status (P = .0053, Fig. 5B). Moreover, lymph node metastasis remained as a significant prognostic factor for HPV-unrelated patients by a univariate analysis (P = .0293, Fig. 5C). The presence of *CDKN2a* mutations was not associated with prognosis in the patients with HPV-unrelated SCCs of the vulva (P = .6930, Fig. 5D).

4. Discussion

In keeping with previous studies [3-5], we have shown that SCCs of the vulva are etiologically different diseases that display distinct clinicopathologic features, genetic alterations, and prognosis. Despite only 12 HPV-related vulvar SCCs in this series, our study revealed *PIK3CA* hotspot mutations, p.Glu545Lys and p.Glu542Lys, in 3 cases. Consistent with these findings, our previous results showed that 30% (3 of 10) of small cell neuroendocrine carcinomas (SCNECs) of the cervix, a high-risk HPV-related (especially HPV 18) cancer harbored *PIK3CA* mutation [18]. In fact, *PIK3CA* is thought to be one of the most frequently mutated genes in HPV-related cancers [19-21]. Despite lacking *PIK3CA* somatic mutation, one tumor harbored *PTEN* (p.Gly129Arg) mutation that is speculated to cause excessive PIP3 at the plasma membrane, and further activate the PI3K/AKT/mTOR pathway that is similarly disrupted by *PIK3CA* mutation [22]. As such, the HPV-related SCCs bearing these mutations, regardless of anatomic site, might be sensitive to mTOR or AKT inhibitor treatment, a potential targeted therapy for this disease. In this study, *FGFR3* p.Ser249Cys mutation was detected in one HPV-related case. Consistent with this finding, a recent study showed that 14% of HPV-positive tumors contained somatic mutations in this gene, raising this possibility of utilization of Inhibitors of FGFR3 as a therapeutic strategy to treat a subset of vulvar SCC patients [9].

In this study, 1 HPV-related vulvar SCCs harbored *TP53* somatic mutations which also contained PIK3CA mutation. Since the high-risk HPV viral oncoprotein E6 has the ability to neutralize the function of p53, the majority of high-risk HPV-related cancers would be expected to have a wild-type *TP53* gene. However, several studies have shown that *TP53* mutation occurred frequently in HPV-related vulvar SCCs. Likewise, we detected *TP53* mutations in HPV-related cervical SCNECs [18]. The role of *TP53* mutation in relation to the presence of high-risk HPV in vulvar SCCs and other HPV-related tumors remain largely unknown, and we speculate these changes involve tumor progression rather than initiating process.

In contrast to high-risk HPV-related SCC of the vulva, HPV-unrelated SCCs harbor a wide variety of somatic *TP53* gene mutations that commonly occur in the early stage of the disease. *TP53* gene mutations have been reported to be present in 30–70% of HPV-negative SCCs of the vulva. Consistently, we detected *TP53* somatic mutations in 15 (88%) of 17 HPV-unrelated tumors, likely due to advanced depth of NGS technology. Similar to previous studies, while the majority of tumors had a single TP53 gene mutation, multiple TP53 mutations had been detected within 1 tumor in 4 cases, suggesting multiple independent genetic events that probably represent tumor heterogeneity. Interestingly, a recent study showed that 14 (82%) of 17 recurrent SCCs of the vulva carried *TP53* gene mutations, five with identical to the mutation in the primary tumors and nine with different, more complex *TP53* gene mutations [23]. This study demonstrated that the majority of *TP53* gene mutated cancers recurred with different *TP53* gene mutations, and a change of *TP53* gene mutational status after >5 years suggests *de novo* oncogenic events/carcinogenesis. They concluded that *TP53* gene mutational status may serve as a prognostic marker for disease-free intervals.

Since *TP53* has been regarded as a caretaker tumor suppressor gene that plays a critical role in conserving stability of genome by preventing mutational alterations, mutations of this gene will lead to genomic instability, aberrant regulation of DNA damage response and apoptosis, and eventually carcinogenesis. Not surprisingly, additional driving events, especially genetic alterations of gatekeeper oncogenes and other caretaker tumor suppressor genes, have been postulated to facilitate the progression of *TP53* mutated precursor lesions to invasive SCC of the vulva. The second most common genetic change detected in this series study was *CDKN2a* somatic mutations that were present in 7 (41%) of 17 cases. Consistent with these findings, *CDKN2a* mutations are considered in the literature as one of the most common molecular alterations in the vulvar SCCs [11,24,25]. *CDKN2a* is a tumor suppressor gene that encodes the proteins p16INK4A and p14ARF that regulate cell cycle progression. One study showed that the combination of mutations in both *TP53* and *CDKN2a* correlated with a significantly worse prognosis in head and neck SCCs [26]. It has been postulated that the tumor cells tend to be more destabilized when both *CDKN2a* and *TP53* are mutated, thus conferring worse prognosis. However, we were unable to verify the *CDKN2a* mutation as a significant prognostic factor due to the small size of this study.

An interesting target identified in this study is BRCA1-associated protein-1 (BAP1) that was mutated in 1 HPV-unrelated and 1 HPV-related SCCs of the vulva. BAP1 is a nuclear deubiquitinating enzyme that regulates transcription,

cell cycle, cell differentiation, DNA damage response and other cellular process [27]. It has been demonstrated that BAP1 can induce radioresistance in HPV-negative head and neck squamous cell cancer (HNSCC) cells and was associated with poor outcomes in patients with HNSCC [28]. Importantly, several studies showed that esophageal and laryngeal SCCs harbored recurrent somatic *BAP1* mutations that may be associated with prognostic significance [28-30]. To the best of our knowledge, this is the first study that demonstrated *BAP1* mutations in vulvar SCCs. A role of BAP1 in the carcinogenic process and prognostic value of vulvar SCCs remains unknown and warrants further investigation.

Similar to previous studies, our results showed that 2 HPV-unrelated tumors contained somatic mutations of HRAS that might be valuable in the development of individualized treatment. One study revealed that vulvar SCC patients with somatic *HRAS*-mutations had a significantly worse prognosis than patients lacking these changes [24]. In our study, although these 2 patients died of disease at a follow-up interval of 3.5 months and 11 months, we cannot assess prognostic value of *HRAS* mutations due to limited number of cases. Mechanistically, signal transduction downstream of oncogenic Ras is mediated through several effectors including the mitogen-activated extracellular kinase (MEK)/extracellular signal regulated kinase (ERK) pathway and PI3K/Akt/mTOR pathway. While the epithelial-mesenchymal transition (EMT) is primarily driven by transcriptional repression of E-cadherin and other epithelial proteins, the MEK/ERK pathway is thought to be indirectly responsible for the process that may confer highly aggressive tumor behavior [31]. Theoretically, a dual blockade of upstream RAS by MEK inhibitors, and a more downstream inhibition of PI3K, may have better clinical results for the vulvar SCC patients with *HRAS* mutations [32].

We have previously reported a *Notch1* mutation in a SCNEC tumor that did not have *PIK3CA* mutation [18]. In the current study, 2 HPV-unrelated tumors also harbored *Notch1* somatic mutations. It has been well documented in the literature that one function of mutant Notch1 is to activate c-Myc and PI3K-AKT-mTOR1 signaling through transcriptional repression of PTEN and promoting growth factor receptor signaling to PI3K-AKT [33]. As such, we speculate the tumors, either SCNECs or vulvar SCCs, bearing these mutations might be sensitive to mTOR or AKT inhibitor treatment, thus providing a potential avenue of targeted therapy for these aggressive disease.

In addition to recurrent somatic mutations, a number of candidate driver genes were identified by clinically reportable threshold from the copy number variations. Of which, 2 HPV-unrelated vulvar SCCs displayed *BIRC3* amplification that was also reported in several human and mouse cancers [34-36]. *BIRC3*, as well as *BIRC2*, are members of the inhibitor of apoptosis family that inhibit apoptosis by interfering with the activation of caspases. In our study, while 1 tumor showed *BIRC3* amplification, the other tumor demonstrated copy number gains including both *BIRC2* and *BIRC3* genes. In fact, these 2 genes are located in the chromosome 11q22 amplicon that is frequently seen in certain cancer types. One study showed 12.9% of cervical carcinomas had a high level amplification of *BIRC2* that was significantly more frequent than *CCND1* amplification (chromosome 11q13 amplicon) [37]. Similar to other findings in this study, we suspect this is the first study showing *BIRC2/3* amplification in vulvar SCCs. The biological and clinicopathological significance of this observation warrants further investigation. The tumor with *BIRC3* amplification also showed copy number gains in other locations containing driver oncogenic genes such as *MYC*, *MCL1*, *CDK4* and *AEPX1*, indicating genetic complexity. Other amplified genes that may play a critical role in tumor initiation and development include *CCND1*, *MYCL*, and *CDK6*. The patients bearing these genetic alterations may benefit from personalized therapy amenable to these targets.

Not surprisingly, we found that lymph node metastasis is a significant prognostic factor despite a limited number of cases in this study. One main aim of this study is to comparatively assess the genetic alterations in primary and metastatic SCCs of the vulva. As a general mechanism, vast majority of cancer deaths including vulvar SCC are caused when cancer spreads from the original tumor to other tissues and organs [38]. However, the mechanisms by which the cancer cells move from the primary tumor, travel through the lymphatic system or the blood, form new tumors in distant location, remain largely unknown. We speculate that specific genetic alterations, acquired in the late stage of tumor development, may confer ability to allow tumor spread. However, while nearly identical genetic changes in primary and metastatic tumors supported a clonal expansion, we failed to identify any specific alterations that may contribute to tumor spread. We suspect genetic alterations such as gene overexpression and/or inactivation, DNA and/or histone-related epigenetic regulation, rather than somatic mutations, may play a critical role in metastasis. Paradoxically, our results showed that four cases with a different molecular abnormality in the primary *versus* metastasis have lost the abnormality in the metastasis. While this may just reflect molecular heterogeneity in the primary, the underlying mechanism by which how this clonal expansion occurs is unclear and warrants further evaluation.

It has been documented in literature that PD-L1 overexpression is readily detected in a substantial proportion of vulvar carcinomas [16,39,40]. Recent studies showed significant discordance of PD-L1 expression in primary and metastatic tumors of certain types [41-45]. We also reason that determination of PD-L1 expression from a single site may not accurately reflect tumor PD-L1 expression status in the different location of vulvar SCCs. Our data demonstrated that PD-L1 expression is spatially discordant between primary and metastatic tumors. In particular, one tumor showed PD-L1 expression has changed from positive to negative. Despite a low case number, our results further highlight the importance of comprehensive assessment of PD-L1 expression in order to avoid false-negative results.

In summary, utilizing a targeted NGS technology, we have identified and confirmed that recurrent *PIK3CA* mutations are commonly present in HPV-related SCCs of the vulva. In contrast, HPV-unrelated primary and metastatic tumors usually harbor somatic mutations of *TP53* and *CDKN2a*. Importantly, identification of novel genetic changes, particularly *BAP1* somatic mutations and *BIRC2/3* amplifications in these tumors, offers the potential for individualized management strategies for treatment of this neglected cancer. Discordant PD-L1 expression in primary and metastatic SCCs of the vulva suggests a novel strategy for checkpoint inhibitor-based immunotherapy.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2019.08.003.

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