original reports

Vulvar Squamous Cell Carcinoma: Comprehensive Genomic Profiling of HPV+ Versus HPV– Forms Reveals Distinct Sets of Potentially Actionable Molecular Targets

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abstract

PURPOSE Vulvar squamous cell carcinoma (vSCC) encompasses two predominant variants: one associated with detectable high-risk strains of human papillomavirus (hrHPV) and a second form often occurring in the context of chronic dermatitis in postmenopausal women. Genomic assessment of a large-scale cohort of patients with aggressive vSCC may identify distinct mutational signatures.

MATERIALS AND METHODS Tumor samples from a total of 280 patients with vSCC underwent hybridization capture with analysis of up to 406 cancer-related genes. Human papillomavirus (HPV) sequences were detected by de novo assembly of nonhuman sequencing reads and aligned to the RefSeq database. Immunohistochemistry for programmed death-ligand 1 (PD-L1) was assessed.

RESULTS One hundred two of 280 vSCCs (36%) contained hrHPV sequences, predominantly HPV 16 (88%). The HPV-positive (HPV+) group was significantly younger (median age, 59 *v* 64 years; *P* = .001). Compared with HPV-negative (HPV–) vSCCs, HPV+ tumors showed more frequent pathogenic alterations in *PIK3CA* (31% *v* 16%; *P* = .004), *PTEN* (14% *v* 2%; *P* < .0001), *EP300* (14% *v* 1%; *P* < .0001), *STK11* (14% *v* 1%; *P* < .0001), *AR* (5% *v* 0%; *P* = .006), and *FBXW7* (10% *v* 3%; *P* = .03). In contrast, HPV– vSCCs showed more alterations in *TP53* (83% *v* 6%; *P* < .0001), *TERTp* (71% *v* 9%; *P* < .0001), *CDKN2A* (55% *v* 2%; *P* < .0001), *CCND1* amplification (22% *v* 2%; *P* < .0001), *FAT1* (25% *v* 4%; *P* < .0001), *NOTCH1* (19% *v* 6%; *P* = .002), and *EGFR* amplification (11% *v* 0%; *P* < .0001), as well as a higher rate of 9p24.1 (*PDL1/PDL2)* amplification (5% *v* 1%) and PD-L1 immunohistochemistry high-positive tumor staining (33% *v* 9%; *P* = .04).

CONCLUSION Comprehensive molecular profiles of vSCC vary considerably with hrHPV status and may inform patient selection into clinical trials. Sixty-one percent of HPV+ vSCCs had a pathogenic alteration in the PI3K/mTOR pathway, whereas HPV– vSCCs showed alterations in *TP53*, *TERTp*, *CDKN2A*, *CCND1*, and *EGFR*, and biomarkers associated with responsiveness to immunotherapy.

JCO Precis Oncol 4:647-661. © 2020 by American Society of Clinical Oncology

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ASSOCIATED CONTENT

Appendix

Author affiliations and support information (if applicable) appear at the end of this article.

Accepted on April 28, 2020 and published at ascopubs.org/journal/po on June 16, 2020: DOI https://doi.org/10.1200/PO.19.00406

INTRODUCTION

Vulvar squamous cell carcinoma (vSCC) comprises > 90% of vulvar cancers and nearly 5% of all gynecologic cancers.[1,2] Radical excision imposes high morbidity, and one third of patients have been shown to experience recurrence after primary treatment.[3] Recent reports have shown durable responses with definitive or neoadjuvant chemoradiation for unresectable cancers.[4,5] For patients with recurrence or distant metastasis, prognosis is poor, with an overall 2-year survival rate of less than 15%.[6] There is a critical need to improve our understanding of the molecular pathogenesis of vSCC to provide insights that may guide more effective therapies.

vSCC develops through two distinct oncogenic pathways. The first major subgroup of vSCC contains detectable integrated or episomal DNA sequences from high-risk strains of human papillomavirus (hrHPV). This subgroup often associates with usual-type vulvar intraepithelial neoplasia (VIN), also known as high-grade squamous intraepithelial lesion.[7] Although other anogenital squamous cell neoplasms have > 85% association with hrHPV infection,[8,9] studies of vSCC report hrHPV infection in a much lower percentage of patients, typically 30%-60%, with substantial variability among studies.[10,11]

CONTEXT

Key Objective

Previous analyses have suggested genetic differences in vulvar squamous cell carcinoma based on human papillomavirus (HPV) status, but restricted sample volume and testing platforms have limited comprehensive identification of statistically significant differences. In a large-scale comparative genomic study, what undiscovered genomic alterations distinguish high-risk HPV-driven versus dystrophic/inflammatory-associated vulvar squamous cell carcinoma?

Knowledge Generated

We identify significantly different molecular profiles based on HPV status. Most high-risk patients with HPV-positive disease demonstrate a pathogenic genomic alteration in the PI3K/mTOR pathway, whereas patients with high-risk HPV-negative disease have enrichment for genomic alterations in *TP53*, *TERTp*, *CDKN2A*, *CCND1*, and *EGFR*, as well as potential predictive biomarkers for responsiveness to immunotherapy.

Relevance

The findings offer insight into vulvar squamous cell carcinoma tumor biology and potential future therapeutic targets.

The second major subtype of vSCC lacks detectable hrHPV sequences, typically arising instead in the context of chronic inflammatory vulvar lesions, particularly lichen sclerosus, and occurs more commonly in older women.[12-14] This vSCC subtype is often preceded by p53-mutant differentiated VIN.[15]

Prior studies of the genomic characteristics of vSCC have consistently identified mutations in *TP53*, with most reporting higher frequencies in human papillomavirus-negative (HPV–) vSCC.[16-20] *PIK3CA* mutations have been reported, but frequencies have varied widely, from 7% to 60%.[17,20] Other reported mutations in vSCC have involved *CDKN2A*, *FBXW7*, *HRAS*, *FAT1*, *FGFR3*, and *PTEN*,[18-21] and copy number analyses have reported *CCND1* and *EGFR* amplifications significantly enriched in HPV– tumors.[22-24] Although these previous analyses have suggested genetic differences between HPV-positive (HPV+) and HPV– vSCC, restricted sample volume and testing modalities have limited comprehensive identification of statistically significant differences.

Given the limited genomic evaluations to date, we performed a search of our archive to identify vSCCs and thoroughly characterized their genome-wide alterations. We identified distinct mutational signatures of the HPV+ and HPV– subgroups of vSCC.

MATERIALS AND METHODS

Cohort and Genomic Analyses

Comprehensive genomic profiling (CGP) was performed in a Clinical Laboratory Improvement Amendments–certified, College of American Pathologists–accredited laboratory (Foundation Medicine, Cambridge, MA). Approval for this study, including a waiver of informed consent and a Health Insurance Portability and Accountability Act waiver of authorization, was obtained from the Western Institutional Review Board (Protocol No. 20152817). The pathologic diagnosis of each cancer was confirmed through review of routine hematoxylin and eosin–stained slides. Sections were macrodissected as necessary to achieve > 20% estimated percent tumor nuclei (100 times the number of tumor cells divided by the total number of all cells with nuclei) in each tumor sample. For genomic analyses, ≥ 60 ng of DNA was extracted from 40-μm sections of 255,008 tumor samples, including 280 vSCC specimens and 1,031 cervical squamous cell carcinoma (cSCC), each from a different patient, in formalin-fixed paraffin-embedded (FFPE) tissue blocks. The samples were assayed by CGP using adaptor ligation, and hybrid capture was performed for all coding exons from 287 (version 1) to 315 (version 2) cancer-related genes plus select introns from 19 (version 1) to 28 (version 2) genes frequently rearranged in cancer (Appendix Table A1). Sequences were analyzed for all classes of genomic alterations (GAs), including short variant alterations, copy number alterations (focal amplifications and homozygous deletions), and select gene fusions or rearrangements, by methods previously described.[25-27] Tumor mutational burden (TMB; mutations/Mb) was determined on 0.8-1.1 megabase pairs of sequenced DNA. Microsatellite instability (MSI) was determined on up to 114 loci. HPV genome sequences were detected by de novo assembly of nonhuman sequencing reads and nucleotide Basic Local Alignment Search Tool (BLASTn) comparison against all viral nucleotide sequences in the National Center for Biotechnology Information RefSeq database. The RefSeq database is comprehensive, and HPV types analyzed for included HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, CP6108, and IS39. HPV types identified in this study were stratified according to the HPV classification described by Muñoz et al,[8] with HPV 16, 18, 31, 33, and 58 labeled hrHPV+ and HPV 6 labeled low risk. HPV 67 was classified as hrHPV+.[28,29] Contigs ≥ 80 nucleotides in length with ≥ 97% sequence identity to the BLAST sequence were required for an HPV+ call.

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Cell-free circulating tumor DNA (ctDNA) was evaluated from blood specimens collected from 10 patients with vSCC ("liquid biopsy") using the hybrid capture-based Illumina Hi-Seq (Illumina, San Diego, CA) technology. Maximum somatic allele frequency was used to estimate the fraction of ctDNA per methods previously described.[30,31]

Mutational Signatures

Mutational signatures were assessed for all tumor samples with at least 20 nondriver somatic missense alterations. Signatures were given by analysis of the trinucleotide context and profiled using the Sanger COSMIC signatures of mutational processes in human cancer.[32] A positive signature required a sample to have at least a 40% fit to a characterized mutational process, including APOBEC overexpression, exposure to ultraviolet light, hypofunction of the BRCA tumor suppressor, and defects in mismatch repair.[32]

Immunohistochemistry

Programmed death-ligand 1 (PD-L1) immunohistochemistry (IHC) was performed regularly in tandem with CGP to guide patient selection for immunotherapy. PD-L1 protein expression was assessed by IHC on 5-micron FFPE tissue sections using the Dako PD-L1 IHC22C3 pharmDx assay (Agilent; Santa Clara, CA; n = 52 vSCCs) or the Ventana (Oro Valley, AZ) PD-L1 (SP142) assay (n = 21 vSCCs), following each manufacturer's instructions. Dako PD-L1 expression was reported as a tumor proportion score, and Ventana PD-L1 was reported as percent tumor area covered by positively staining tumor cells and immune cells. Less than 1% staining was defined as negative, 1%-49% was defined as low positive, and ≥ 50% was defined as high positive.

Clinicopathologic Analysis of the vSCC Cohort

A total of 280 vSCCs were assayed with CGP (Foundation Medicine), using material sent from treating institutions, from 2014 to 2019. Human investigations were performed after approval by a local human investigations committee and in accordance with an assurance filed with and approved by the Department of Health and Human Services, where appropriate. Clinicopathologic data were extracted from the accompanying pathology report. Two board-certified anatomic pathologists (E.A.W. and D.I.L.) reviewed histopathology of representative sections.

Categorical data were analyzed using the Fisher exact test owing to the size of the cohort. The Mann-Whitney *U* test was used for comparisons of TMB. A 2-tailed *P* value of < .05 was considered statistically significant.

RESULTS

Clinicopathologic Features

From an internal series of 255,008 patient tumors that had undergone prior hybrid capture-based DNA sequencing, 280 vSCCs, each from a different patient, were identified. All patients had clinically advanced/metastatic disease at the time of sequencing. Ages ranged from 25 to 92 years, with a median age of 62 years. Sequencing was performed on the primary tumor in 200 patients and on metastases in 80 (57 regional lymph nodes and 23 distant sites).

HPV status and typing were determined on all 280 patient samples; 102/280 vSCCs (36%) contained hrHPV sequences, predominantly HPV 16 (88%; Table 1). For low-risk HPV, two patient samples had HPV 6 sequences, including one patient sample with concurrent HPV 16 and HPV 33. Patients were significantly younger in the HPV+ group than in the HPV– group (median age, 59 *v* 64 years; *P* = .001).

For the 102 hrHPV+ vSCCs, 62 were sequenced using the original primary tumors and 40 from metastatic site biopsies (13 distant, including six lung, two distant lymph nodes, one liver, one brain, one chest wall, one pleura, and one retroperitoneum). The single low-risk HPV+ vSCC without concurrent hrHPV was sequenced using original primary tumor. For the 177 HPV– vSCCs, 137 were sequenced using the original primary tumors and 40 from metastatic site biopsies (10 distant, including seven lung, one pleural, one abdominal, and one to bone).

Comprehensive Genomic Profiling

Figure 1 displays the distribution of GAs by HPV status. The overall prevalence of 1 or more known oncogenic GAs in the cohort was 98%, including 95% of HPV+ vSCCs and 99% of HPV– vSCCs. The percentage of each GA in the cohort overall is included in Appendix Figure A1.

We compared the rate of oncogenic mutations between HPV+ and HPV– vSCCs. Alterations frequently observed in HPV+ tumors (Table 2) differed from those observed in HPV– tumors (Table 3). The majority of *CCND1*-amplified vSCCs (n = 41 total) showed amplification of other genes at 11q13, including *FGF3* (n = 40), *FGF19* (n = 40), and *FGF4* (n = 36). The only specific point mutation with a significant difference between HPV+ and HPV– was *PIK3CA* E545K, an activating mutation that was significantly enriched in HPV+ vSCCs (Table 2). The percentage

TABLE 1. Oncogenic HPV Typing

HPV Subtype	No. of vSCCs
16[a]	90
18	7
31	1
33[a]	3
58	1
67	1
6[a]	2

Abbreviation: HPV, human papillomavirus; vSCC, vulvar squamous cell carcinoma.

[a]A single vSCC showed concurrent HPV 6, HPV 16, and HPV 33 genomic sequences.

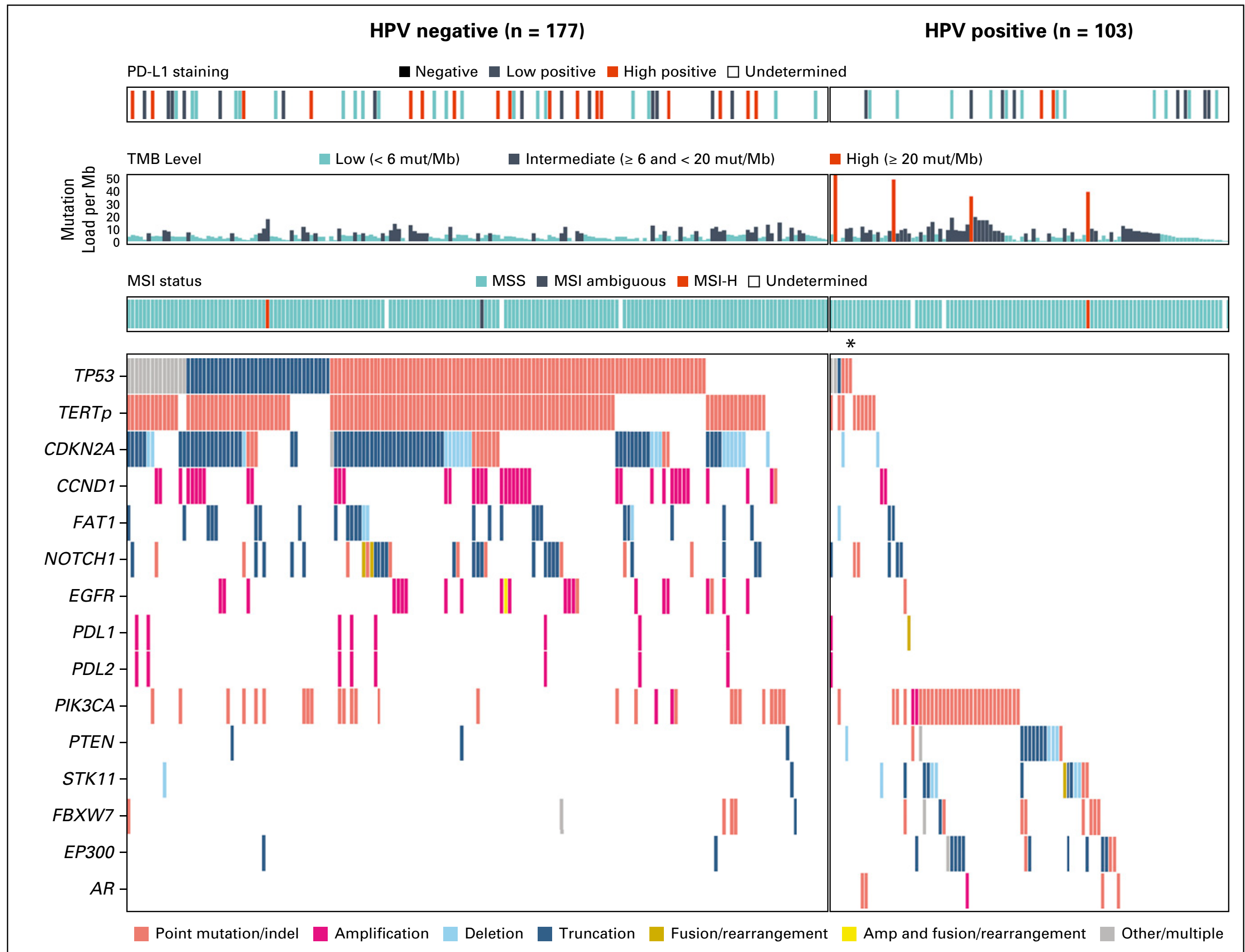

FIG 1. Tile plot summary of pathogenic molecular alterations in vulvar squamous cell carcinoma based on human papillomavirus status. (*) A single low-risk only HPV+ vSCC is denoted by an asterisk. amp, amplification; Mb, megabase; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSS, microsatellite stable; mut, mutation; PD-L1, programmed death-ligand 1; TMB, tumor mutation burden.

of each GA in the HPV– and HPV+ cohorts is included in Appendix Figures A2A and A2B, respectively.

Frequencies of specific biomarkers associated with responsiveness to immunotherapy differed between the vSCC subgroups (Fig 2). Of the 73 vSCCs for which PD-L1 IHC was performed, a higher rate of PD-L1 IHC high-positive tumor staining was identified in HPV– versus HPV+ vSCC (33% *v* 9%; *P* = .04; Figs 2 and 3). No significant correlation was identified between PD-L1 IHC and other GAs or biopsy site.

Although the median TMB of HPV+ vSCC was significantly higher overall than that of HPV– disease (6.1 *v* 3.8; *P* = .008), a complicating factor was the higher percentage of HPV– vSCCs sequenced from the primary tumor (77% [137/177] HPV– *v* 61% [63/103] HPV+). For vSCCs sequenced from primary tumor only, the median TMB was still significantly higher for HPV+ versus HPV– tumors (6.1 versus 3.8; *P* = .0178). A single MSI-high vSCC was present in the entire cohort: an HPV 16(+) vSCC with an *MLH1* splice site mutation.

In HPV+ tumors, a higher rate of *STK11* GAs was observed in metastases than in primary sites (23% *v* 8%; *P* = .043). In HPV– tumors, a higher rate of *SMAD4* GAs was observed in metastases than in primary sites (15% v 4%; *P* = .0295). All other GAs, as well as age and PD-L1 IHC staining, showed no significant differences between primary versus metastatic samples controlled for HPV status.

Comparison of HPV 16 with other hrHPV subtypes revealed no significant differences in demographics, TMB, or sequenced site. Non-HPV 16 hrHPV tumors did show enrichment for *TERTp* mutations (33% *v* 6%; *P* = .0075). This finding was similar to that seen in the cSCC cohort (23% *v* 10%; *P* < .0001). Of note, a higher percentage of patients with HPV 16 disease was present in the HPV+ vulvar cohort as compared to the HPV+ cervix cohort (88% *v* 65%; *P* < .0001).

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TABLE 2. Mutation Percent Frequency, by HPV Status, of GAs That Were More Frequent in the HPV+ Cohort, With *P* Value

Functional Class of Mutated Genes	Mutation	HPV Status (%) HPV+	HPV–	*P*
PI3K/AKT/mTOR pathway (61% of HPV+ *v* 27% of HPV–; ***P* < .0001**)	***PIK3CA***	**31.1**	**15.8**	**.004**
	***PIK3CA* E545K**	**14.6**	**2.8**	**.0004**
	***KMT2D*[a]**	**15.5**	**7.3**	**.04**
	PTEN	**13.6**	**1.7**	**< .0001**
	STK11	**13.6**	**1.1**	**< .0001**
	FBXW7	**9.7**	**3.4**	**.03**
	***SOX2* amp[a]**	**4.9**	**1.1**	**.0267**
	PIK3R1	2.9	0.6	.14
	AKT1	1.9	0.6	.56
	MTOR	1.9	1.1	.63
Epigenetic regulation	***EP300***	**13.6**	**1.1**	**< .0001**
	BAP1	**4.9**	**0.6**	**.03**
	PBRM1	5.8	1.7	.08
	KDM6A	6.8	2.3	.11
	KMT2C	6.8	3.7	.49
	ARID1A	2.9	2.3	.71
Cell cycle regulation	*RB1*	5.8	1.7	.08
Transcriptional regulation	*CDK12* inactivating	5.8	1.1	.055
	AR	**4.9**	**0**	**.006**
Receptor tyrosine kinase	*FGFR3*	2.9	1.1	.36

NOTE. $P < .05$ are indicated with bold type.
Abbreviations: +, positive; –, negative; amp, amplification; GAs, genomic alterations; HPV, human papillomavirus.
[a]Limited data in literature on role in PI3K/AKT signaling.

For mutational signatures, 261 vSCCs were available for analysis. Thirty-three (12.6%) were identified with an APOBEC (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) signature (12 HPV+ and 21 HPV–), two with BRCA signature (one HPV+, one HPV–), seven with mismatch repair (two HPV+, five HPV–), and a single tumor with ultraviolet signature (HPV–). No vSCCs showed any mutational signature characteristic of cancer therapies, such as alkylating agents.

Eight patients had at least two separate specimens analyzed. Of these eight patients, blood specimens ("liquid biopsies") were evaluated for ctDNA in four patients and additional tissue biopsies in four patients. Three of four liquid biopsies showed at least one pathogenic GA present in the associated tissue biopsy (Appendix Table A2). Separate from our 280 patients in the vSCC cohort, ctDNA was evaluated on six patients with known vSCC but without tissue biopsy sequencing data. GAs were detected in five of six of these patients (Appendix Table A2).

HPV+ cSCC (n = 864) showed GAs that were largely similar to what we found in HPV+ vSCC (n = 103; Appendix Table A3). Although low in frequency, GAs in *KDM6A*, *AR*, and *CDK12* were significantly higher in vSCC versus cSCC (Appendix Table A3).

DISCUSSION

In this study, hybrid capture-based DNA sequencing was applied to a large series of patient tumors to better characterize the genomic landscape of vSCC and to identify important genetic differences between HPV+ and HPV– disease. Consistent with prior studies, a high rate of mutation was identified overall, with 98% of tumors in the analysis containing one or more known oncogenic mutations.[17,18]

Mutational profiles sharply differentiated HPV+ and HPV– disease. HPV+ vSCC showed mutations in the PI3K/mTOR pathway, with 61% of tumors containing GAs in the pathway, with the majority of GAs showing significant association with HPV+ status (Table 2). Conversely, HPV– vSCC was defined by GAs in *TP53*, *TERTp*, *CDKN2A*, *CCND1*, *FAT1*, *NOTCH1*, and *EGFR* (Table 3). In addition, HPV+ vSCC sequencing results from primary tumors showed significantly higher TMB than results from HPV– primary-sequenced tumors, and HPV– vSCCs showed a significantly higher rate of PD-L1 IHC high-positive tumor staining.

Prior reports of GAs in vSCC have identified trends in mutations between HPV+ and HPV– types, although there has been substantial overlap in mutational profile, possibly owing to limited case volume.[16-18,20] Weberpals et al[18]

TABLE 3. Mutation Percent Frequency, by HPV Status, of GAs That Were More Frequent in the HPV– Cohort, With *P* Values

		HPV Status (%)		
Functional Class of Mutated Genes	**Mutation**	**HPV+**	**HPV–**	***P***
DNA damage	***TP53***	**5.8**	**82.5**	**< .0001**
Cell cycle regulation	***TERTp***	**8.7**	**71.2**	**< .0001**
	CDKN2A	**1.9**	**54.8**	**< .0001**
	***CCND1* amp**	**1.9**	**22**	**< .0001**
	CDK6	0	2.3	.3
Single pass transmembrane receptor	***FAT1***	**4.1**	**25**	**< .0001**
	NOTCH1	**5.8**	**19.2**	**.002**
Apoptosis	*CASP8*	4.9	11.9	.06
Receptor tyrosine kinase	***EGFR* amp**	**0**	**11.3**	**< .0001**
	ERBB2	1.9	2.3	1
Transcription factor (TGF-β signaling)	*SMAD4*	1.9	6.8	.09
RAS/MAPK pathway	*HRAS*	2.9	6.2	.27
	KRAS	1.9	2.3	1
	NF1	2.9	4	.75
Transcriptional regulation	*NFE2L2*	2.9	7.3	.18
Immune regulation	*PDL1* amp	1	4.5	.16
	PDL2 amp	1	4.5	.16
Epigenetic regulation	*ARID2*	0	3.7	.15

NOTE. $P < .05$ are indicated with bold type.

Abbreviations: +, positive; –, negative; amp, amplification; GAs, genomic alterations; HPV, human papillomavirus.

described a series of 43 patients with vSCCs sequenced with a 50-gene panel. HPV+ vSCC tended to be enriched in *FGFR3* and *PTEN* mutations, although the rates were not statistically distinguishable from the HPV– vSCC. The HPV– tumors demonstrated significant enrichment for *TP53* mutations and showed a nonsignificant tendency toward more *HRAS*, PI3K, and *CDKN2A* mutations. Choschzick et al[22] specifically examined *CCND1* copy number changes in 183 vSCCs and identified amplifications in 22%, with a significant association with HPV– tumors.[23] Growdon

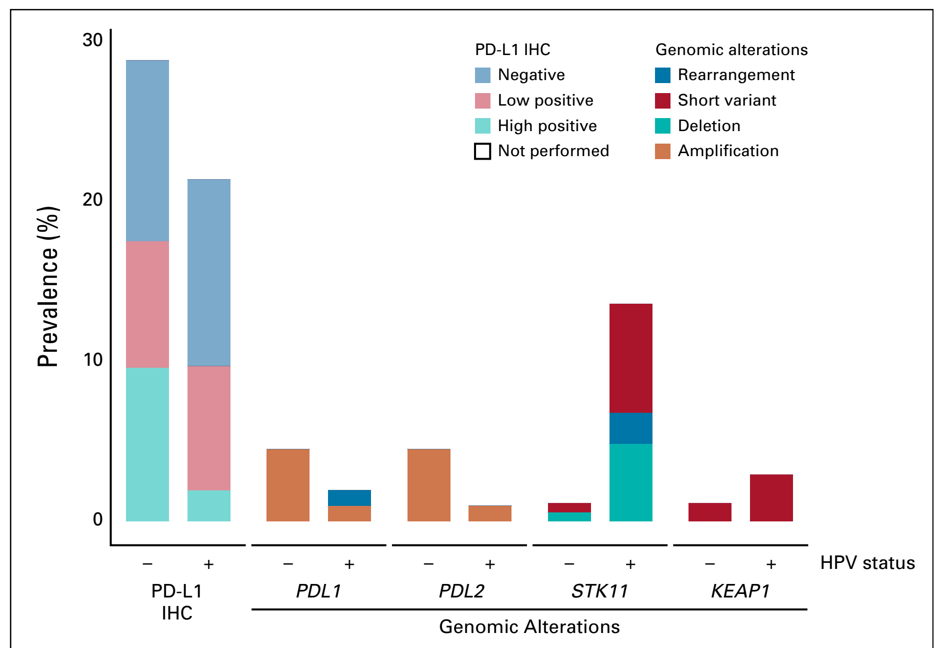

FIG 2. Frequencies of specific biomarkers associated with response to immunotherapy between the vulvar squamous cell carcinoma subgroups. Of the 73 vulvar squamous cell carcinoma for which PD-L1 IHC was performed, 33% of HPV-negative and 9% of HPV-positive were PD-L1 tumor high-positive; $P = .04$. HPV, human papillomavirus; IHC, immunohistochemistry; PD-L1, programmed death-ligand 1.

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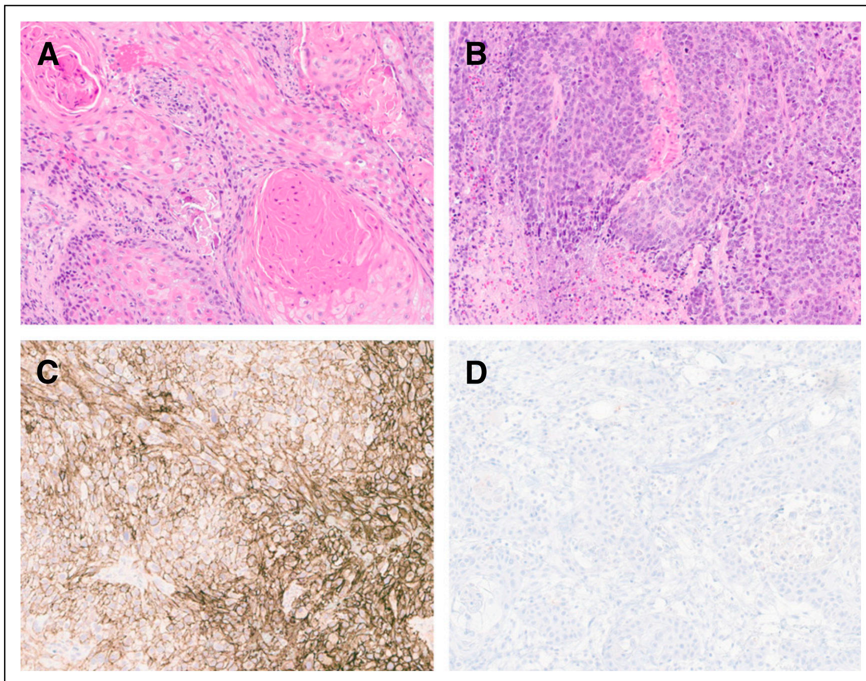

FIG 3. Histopathology of vulvar squamous cell carcinoma ranged from (A) well differentiated with abundant keratin to (B) poorly differentiated (hematoxylin and eosin stains, 400×). (C) Programmed death-ligand 1 (PD-L1) staining of human papillomavirus (HPV)–negative vSCCs showed significantly higher frequency of high-positive tumors, whereas (D) HPV-positive disease was largely negative for PD-L1 stain (PD-L1 immunohistochemistries, 400×).

et al[24] evaluated *EGFR* amplification in 51 vSCCs, and identified amplification in 12% of tumors, with significant association with poor prognosis and HPV– status. Zięba et al[19] performed sequencing of 81 vSCCs with a 50-gene panel, and the results differed from other studies, most strikingly in the absence of clear genomic differences between HPV+ and HPV– disease. The authors reported *TP53* and *CDKN2A* mutations in both HPV+ and HPV– vSCC, whereas mutations in *PIK3CA*, *FBXW7*, *HRAS*, *FGFR3*, *STK11*, *AKT1*, *SMAD4*, and *PTEN* were found at low frequencies in both types of vSCC.[19] Zięba et al[19] noted, however, that the 2 HPV tests that they used gave highly inconsistent results and that those HPV tests had not been developed for analyzing tissue-derived DNA.[19] These difficulties in identifying HPV+ and HPV– disease may account for the divergence of their results from several prior studies.[16-18,20] In our study, HPV status clearly divided our large cohort into two significantly different genomic-defined diseases.

PI3K/mTOR pathway mutations, including *STK11,* a negative regulator of mTOR signaling, have been described in a wide range of HPV-driven cancers.[33,34] In our cohort, a significantly higher rate of *STK11* GAs was observed in HPV+ tumors sequenced from metastases, compared with HPV+ tumors sequenced from the primary site. *STK11* has been previously correlated with poor response to anti–programmed death-1 therapy in *KRAS*-mutant lung adenocarcinoma.[35] It is conceivable that a similar role could exist in HPV+ vSCC as a putative tumor immune-escape mechanism, but additional studies are needed.

A minority of vSCCs showed distinctive mutational signatures. The most common pattern was the APOBEC signature seen in 33 vSCCs, including 12 with HPV+ and 21 with HPV– disease. This signature reflects APOBEC cytidine deaminase DNA-editing activity[36,37] and has been noted to be important in development of thoracic cancers, with possible implications for predicting response to immunotherapy.[38,39]

Several of the GAs observed to be significantly enriched in the HPV– cohort are in pathways functionally relevant to HPV pathogenesis. *TP53*, *TERT*, and *CDKN2A* are deregulated by HPV E6 and/or E7, whereas *EGFR* recycling is altered by HPV E5.[40-42] Beyond specific GAs, key differences were identified in TMB and PD-L1 IHC staining patterns. HPV induces genomic instability, which may account for the increased TMB in the primary HPV+ cohort.[43] In addition, HPV infection reduces the cellular immune response by decreasing the interferon antiviral response.[44]

CGP may reveal opportunities for targeted therapies to be tested in clinical trials. The similarity of the genomic profile of HPV+ vSCC to that of other HPV+ gynecologic cancers (eg, cSCC) may allow for adaptation of therapies. Although HPV vaccines may reduce rates of cancer over the long term, HPV+ cancers including vSCC will persist in the near term.[45]

Results of clinical trials against the PI3K/AKT/mTOR pathway in HPV-related neoplasms have shown limited benefit, with an unclear role in treatment of cSCC.[46,47] Additional evaluation through selection of appropriate patients and use of more potent or combination therapy may be a helpful strategy.[48] The activating point mutation *PIK3CA* E545K was significantly enriched in HPV+ vSCC; analysis of specific mutations as part of clinical trials may

improve stratification of therapeutic sensitivities. KMT2D, an epigenetic modifier, and transcription factor SOX2 can activate and interact with the PI3K pathway.[49-51] GAs in both are enriched in HPV+ vSCC (Table 2). Tumors with GAs in *KMT2D* may be sensitive to Aurora kinase inhibition.[52]

In light of the many recent successes of immune checkpoint inhibitors, a careful approach to patient selection for clinical trials of these agents may be valuable. In our cohort, HPV– vSCCs showed a significantly higher rate of PD-L1 IHC high-positive tumor staining, a higher rate of *PDL1* amplification, and significantly lower rates of *STK11* alterations. *SMAD4*, a regulator of the TGF-β signaling pathway, was inactivated at a significantly higher frequency in HPV– sequenced metastases (*v* primary sites). vSCCs in this category may benefit from novel targeted therapeutics.[53]

Other identified potential therapeutic targets include GAs in receptor tyrosine kinases, cell cycle regulation, and the MAPK pathway. Early work in GAs that affect epigenetic regulation indicates EZH2 inhibitors may be a viable therapeutic strategy.[54,55]

Our study also provides a proof of concept that liquid biopsy detects ctDNA in vSCC, with three of four demonstrating at least one pathogenic GA detected in the tissue biopsy from the same patient. Liquid biopsy may be a valuable method in vSCC, and additional investigation is warranted.

Limitations in the study include the distinct patient population. Tumor samples undergoing CGP are usually sent by clinicians seeking targeted therapy for patients with advanced disease. An additional limitation is the inadequate data on treatment history of the patients before tumor sequencing; controls for TMB and resistance GAs that may have arisen from local radiation or systemic treatment were not available. Future work is needed to correlate genetic findings with treatment exposure and follow-up data, which are not included in this study.

In this study, we provided evidence that HPV+ and HPV– vSCC are two distinct diseases, each with a characteristic molecular profile. Biomarker identification and HPV status will be critical to inform stratification in clinical trials. Our findings provide compelling rationale for tandem CGP and HPV assessment of advanced vSCCs to more fully inform potential therapeutic options.

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PRIOR PRESENTATION

Presented at the AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, Boston, MA, October 26-30, 2019.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The following represents disclosure information provided by authors of this manuscript. All relationships are considered compensated unless otherwise noted. Relationships are self-held unless noted. I = Immediate Family Member, Inst = My Institution. Relationships may not relate to the subject matter of this manuscript. For more information about ASCO's conflict of interest policy, please refer to www.asco.org/rwc or ascopubs.org/po/author-center.

Open Payments is a public database containing information reported by companies about payments made to US-licensed physicians (Open Payments).

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No other potential conflicts of interest were reported.

ACKNOWLEDGMENT

We thank our colleagues for their helpful comments during the preparation of this article.

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APPENDIX

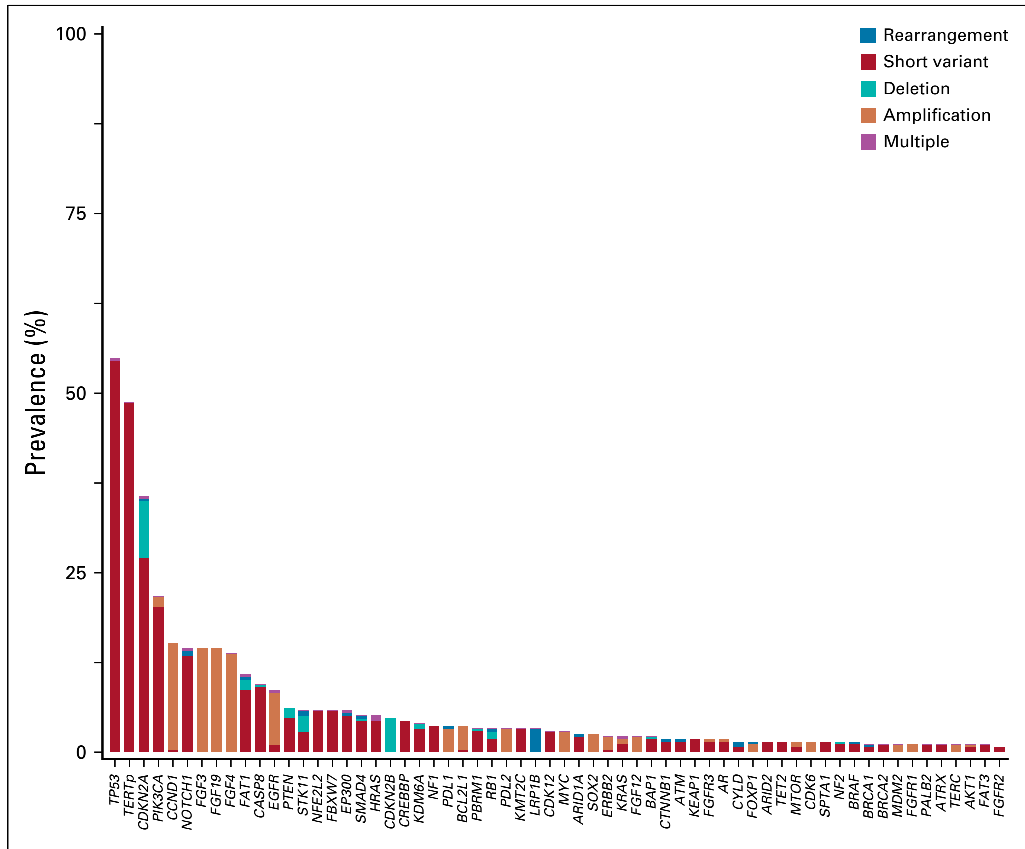

FIG A1. Percentage of each genomic alteration in the vulvar squamous cell carcinoma cohort overall.

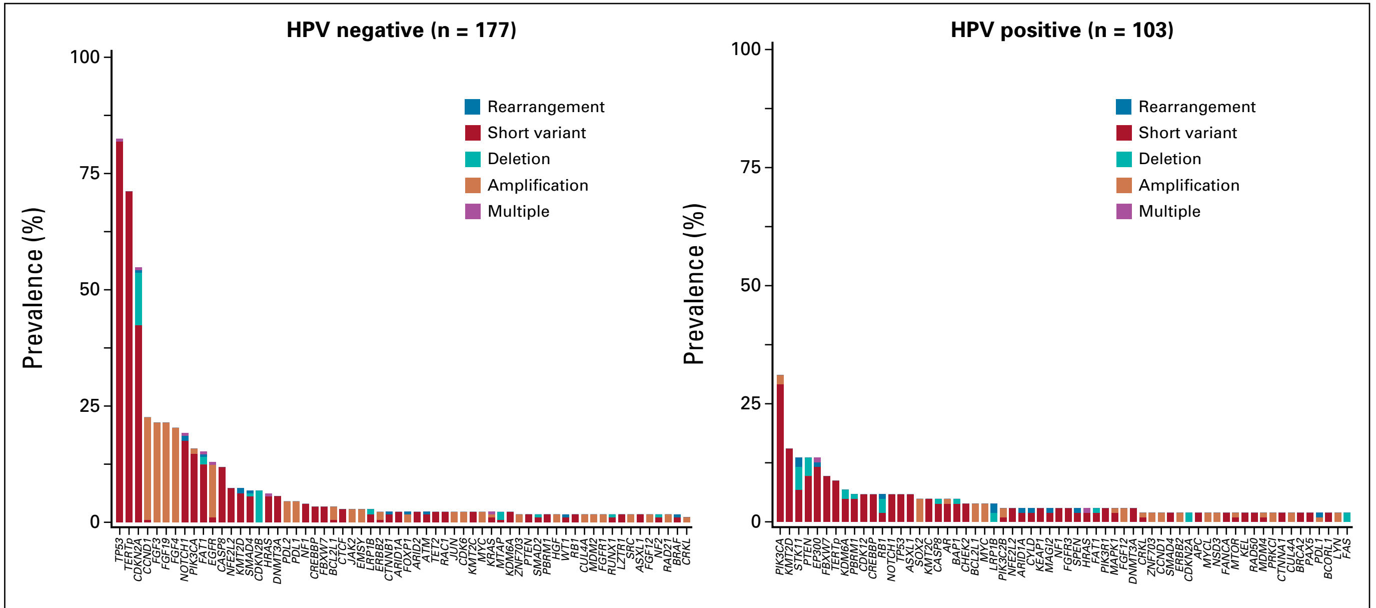

FIG A2. Percentage of each genomic alteration in the (A) human papillomavirus (HPV)-negative vulvar squamous cell carcinoma cohort and the (B) HPV-positive cohort.

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TABLE A1. Complete list of all sequenced genes in the FoundationOne platform.

Genes With Full Coding Exonic Regions for Detection of Substitutions, Indels, and Copy Number Alterations																			
ABL1	*ACVR1B*	*AKT1*	*AKT2*	*AKT3*	*ALK*	*AMER1*	*APC*	*AR*	*ARAF*	*ARFRP1*	*ARID1A*	*ASXL1*	*ATM*	*ATR*	*ATRX*	*AURKA*	*AURKB*	*AXL*	*BAP1*
BARD1	*BCL2*	*BCL2L1*	*BCL2L2*	*BCL6*	*BCOR*	*BCORL1*	*BRAF*	*BRCA1*	*BRCA2*	*BRD4*	*BRIP1*	*BTG1*	*BTG2*	*BTK*	*C11orf30*	*CALR*	*CARD11*	*CBFB*	*CBL*
CCND1	*CCND2*	*CCND3*	*CCNE1*	*CD22*	*PDL1*	*CD70*	*CD79A*	*CD79B*	*CDC73*	*CDH1*	*CDK12*	*CDK4*	*CDK6*	*CDK8*	*CDKN1A*	*CDKN1B*	*CDKN2A*	*CDKN2B*	*CDKN2C*
CEBPA	*CHEK1*	*CHEK2*	*CIC*	*CREBBP*	*CRKL*	*CSF1R*	*CSF3R*	*CTCF*	*CTNNA1*	*CTNNB1*	*CUL3*	*CXCR4*	*DAXX*	*DDR2*	*DNMT3A*	*DOT1L*	*EED*	*EGFR*	*EP300*
EPHA3	*EPHB1*	*ERBB2*	*ERBB3*	*ERBB4*	*ERG*	*ERRFI1*	*ESR1*	*EZH2*	*FAM123B*	*FAM46C*	*FANCA*	*FANCC*	*FANCG*	*FANCL*	*FAS*	*FBXW7*	*FGF10*	*FGF14*	*FGF19*
FGF23	*FGF3*	*FGF4*	*FGF6*	*FGFR1*	*FGFR2*	*FGFR3*	*FGFR4*	*FH*	*FLCN*	*FLT1*	*FLT3*	*FOXL2*	*FUBP1*	*GABRA6*	*GATA3*	*GATA4*	*GATA6*	*GID4*	*GNA11*
GNA13	*GNAQ*	*GNAS*	*GRM3*	*GSK3B*	*H3F3A*	*HDAC1*	*HGF*	*HNF1A*	*HRAS*	*HSD3B1*	*ID3*	*IDH1*	*IDH2*	*IGF1R*	*IKBKE*	*IKZF1*	*INPP4B*	*IRF2*	*IRF4*
IRS2	*JAK1*	*JAK2*	*JAK3*	*JUN*	*KDM5A*	*KDM5C*	*KDM6A*	*KDR*	*KEAP1*	*KEL*	*KIT*	*KLHL6*	*KMT2A*	*KMT2D*	*KRAS*	*LYN*	*MAF*	*MAP2K1*	*MAP2K2*
MAP2K4	*MAP3K1*	*MAPK1*	*MCL1*	*MDM2*	*MDM4*	*MED12*	*MEF2B*	*MEN1*	*MET*	*MITF*	*MLH1*	*MMSET*	*MPL*	*MRE11*	*MRE11A*	*MSH2*	*MSH3*	*MSH6*	*MTAP*
MTOR	*MUTYH*	*MYC*	*MYCL*	*MYCN*	*MYD88*	*NF1*	*NF2*	*NFE2L2*	*NFKBIA*	*NKX2-1*	*NOTCH1*	*NOTCH2*	*NOTCH3*	*NPM1*	*NRAS*	*NSD2*	*NSD3*	*NT5C2*	*NTRK1*
NTRK2	*NTRK3*	*P2RY8*	*PALB2*	*PARK2*	*PAX5*	*PBRM1*	*PDCD1*	*PDL2*	*PDGFRA*	*PDGFRB*	*PDK1*	*PIK3C2B*	*PIK3CA*	*PIK3CB*	*PIK3R1*	*PIM1*	*PMS2*	*POLD1*	*POLE*
PPP2R1A	*PRDM1*	*PRKAR1A*	*PRKCI*	*PRKN*	*PTCH1*	*PTEN*	*PTPN11*	*PTPRO*	*QKI*	*RAC1*	*RAD21*	*RAD51*	*RAF1*	*RARA*	*RB1*	*RBM10*	*RET*	*RICTOR*	*RNF43*
ROS1	*RPTOR*	*SDHA*	*SDHB*	*SDHC*	*SDHD*	*SETD2*	*SF3B1*	*SGK1*	*SMAD2*	*SMAD4*	*SMARCA4*	*SMARCB1*	*SMO*	*SNCAIP*	*SOCS1*	*SOX2*	*SOX9*	*SPEN*	*SPOP*
SRC	*STAG2*	*STAT3*	*STK11*	*SUFU*	*SYK*	*TBX3*	*TERC*	*TET2*	*TGFBR2*	*TNFAIP3*	*TNFRSF14*	*TP53*	*TSC1*	*TSC2*	*U2AF1*	*VEGFA*	*VHL*	*WHSC1*	*WHSC1L1*
WT1	*WTX*	*XPO1*	*ZNF217*	*ZNF703*															

Genes With Select Intronic Regions for the Detection of Gene Rearrangements																			
ALK	*BCL2*	*BCR*	*BRAF*	*BRCA1*	*BRCA2*	*EGFR*	*ETV4*	*ETV5*	*ETV6*	*EWSR1*	*FGFR1*	*FGFR2*	*FGFR3*	*KIT*	*KMT2A*	*MSH2*	*MYB*	*MYC*	*NOTCH2*
NTRK1	*NTRK2*	*NUTM1*	*PDGFRA*	*RAF1*	*RARA*	*RET*	*ROS1*	*SLC34A2*	*TERT*	*TMPRSS2*									

TABLE A2. Liquid Biopsy Results With Available Paired Specimens

	Blood/F1L Results[a]	Prior Paired Specimen Results (if available)					
Patient No.	Short Variants (% reads)	Specimen Type/ Testing	Short Variants	Copy Number Alterations	Rearrangements	HPV Reads	Time to Liquid Biopsy
1	***TERT* c.-124C>T (0.82); *CDKN2A* p.splice site 151-3_151delCAGG (0.61)**; *TP53* p.C176F (0.84); *ATM* p.M557fs*9 (1.19)	Primary site/ F1CDx	***TERT* c.-124C>T; *CDKN2A* p.splice site 151-3_151delCAGG**; *TP53* R282W; *ATM* R337C; *NOTCH1* L1834fs*49; *CCNE1* R95W	*PDL2* amp; *PDL1* amp		Negative	2 days
2	***STK11* p.R304W (1.21)**; *GNAS* p.R201C (6.45)	Regional LN/ F1CDx	***STK11* p.R304W**; *FBXW7* p.R479Q; *EED* p.R414*			HPV-16	1 month
3	***TP53* p.R175H (3.57)**; *CDH1* p.R335* (0.42)	Regional LN/ F1CDx	***TP53* p.R175H**; *PBRM1* p.Q170*	*MYC* amp; *RUNX1* loss; *CDKN2A* loss; *CCND1/FGF4/FGF3/FGF19* amp		Negative	9 months
4	Negative	Primary site/ F1CDx	*TP53* p.G244D; *TERT* c.-124C>T; *CDKN2A* p.L32_L37del; *RUNX1* p.S276fs*324; *NF1* p.K1942*		*WT1-PAX6*	Negative	11 months
5	*TP53* p.V172F (0.13); *TP53* p.G266R (0.12)	Blood/F1L	Negative			N/A	7 months
6	*PTEN* p.R335* (1.56); *TP53* p.G244D (1.44); *CDKN2A* p.R80* (0.59); *TERT* c.-124C>T (0.26)						
7	*TP53* p.I195F (0.90); *TP53* p.C176S (0.57); *TERT* c.-124C>T (0.38)						
8	*PTEN* p.V85fs*7 (8.06); *TP53* p.R213Q (5.28)						
9	*TP53* p.Y220N (0.27)						
10	Negative						

NOTE. Matching genomic alterations are in bold.

Abbreviations: amp, amplification; HPV, human papillomavirus; N/A, not applicable.

[a]No copy number alterations or rearrangements were detected by liquid biopsy in these patients.

TABLE A3. Mutation Percent Frequency in vSCC Versus cSCC, With *P* Values

Functional Class of Mutated Genes	Mutation	HPV+ vSCC (%)	HPV+ cSCC (%)	*P*
PI3K/AKT/MTOR pathway	*PIK3CA*	31.1	36.7	.279
	PIK3CA E545K	14.6	19.6	.287
	*KMT2D**	15.5	15.7	1.00
	PTEN	13.6	11	.412
	STK11	13.6	11.1	.416
	FBXW7	9.7	14.2	.229
	*SOX2**	4.9	5.2	1.00
	PIK3R1	2.9	3.6	1.00
	AKT1	1.9	2.7	1.00
	MTOR	1.9	1.0	.33
Epigenetic regulation	*EP300*	13.6	10.0	.235
	BAP1	4.9	7.4	.422
	PBRM1	5.8	2.6	.109
	KDM6A	6.8	2.5	.0274[a]
	KMT2C	6.8	6.7	1.00
	ARID1A	2.9	4.9	.618
Cell cycle regulation	*TERTp*	8.7	14.5	.1297
	RB1	5.8	5.7	1.00
Transcriptional regulation	*AR*	4.9	0.2	.0002[a]
	CDK12	5.8	1.3	.006[a]
Receptor tyrosine kinase	*FGFR3*	2.9	4.1	.7895
	ERBB2	1.9	5.6	.156
	EGFR amp	0	2.2	.25

Abbreviations: amp, amplification; cSCC, cervical squamous cell carcinoma; HPV, human papillomavirus; vSCC, vulvar squamous cell carcinoma.

[a] $P < .05$.