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ORIGINAL RESEARCH ARTICLE

TP53 mutation and human papilloma virus status as independent prognostic factors in a Norwegian cohort of vulva squamous cell carcinoma

Harsh Nitin Dongre[1] | Rammah Elnour[1] | Stian Tornaas[1] | Siren Fromreide[1] | Liv Cecilie Vestrheim Thomsen[2,3] | Ingrid Benedicte Moss Kolseth[3] | Elisabeth Sivy Nginamau[4] | Anne Christine Johannessen[1,4] | Olav Karsten Vintermyr[1,4] | Daniela Elena Costea[1,4] | Line Bjørge[2,3]

[1]Center for Cancer Biomarkers CCBIO and Gade Laboratory of Pathology, Department of Clinical Medicine, University of Bergen, Bergen, Norway

[2]Center for Cancer Biomarkers CCBIO, Department of Clinical Science, University of Bergen, Bergen, Norway

[3]Department of Obstetrics and Gynecology, Haukeland University Hospital, Bergen, Norway

[4]Department of Pathology, Laboratory Clinic, Haukeland University Hospital, Bergen, Norway

Correspondence
Line Bjørge, Department of Obstetrics and Gynecology, Haukeland University Hospital, Jonas Lies vei 72, N-5058, Bergen, Norway
Email: line.bjorge@uib.no

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Abstract

Introduction: Vulva squamous cell carcinoma (VSCC) develops through two separate molecular pathways—one involving high-risk human papilloma virus infection (HPV-associated), and the other without HPV infection (HPV-independent) often involving *TP53* mutation. HPV-associated VSCC generally has a better progression-free survival than HPV-independent VSCC. The aim of this study was to determine *TP53* mutation status using immunohistochemistry, compare different methods of HPV detection and correlate both with survival in a retrospective cohort of 123 patients with VSCC.

Material and methods: Immunohistochemistry for p53, Ki67 and $p16^{INK4A}$ (a surrogate marker for HPV infection) was performed on formalin-fixed paraffin-embedded tissues from a cohort of surgically treated VSCC patients to identify molecular subtypes of VSCC. Presence of HPV infection was detected by HPV DNA PCR and HPV mRNA in situ hybridization (ISH). The Pearson chi-square test and multivariable Cox regression model were used to investigate the association of different parameters with progression-free survival and disease-specific survival (DSS), and Kaplan–Meier curves were used to show the association of different parameters with survival.

Results: The results of p53 and $p16^{INK4A}$ immunohistochemistry confirmed three VSCC subtypes associated with different prognosis. The *TP53* mutation status was identified as an independent prognostic factor of worse progression-free survival ($p = 0.024$) after adjustment for FIGO stage. $p16^{INK4A}$ immunohistochemistry, mRNA ISH, and DNA PCR had excellent concordance in terms of HPV detection. According to the multivariable Cox regression model, the presence of hrHPV mRNA correlated

Abbreviations: *EGFR*, epidermal growth factor receptor; FFPE, formalin-fixed paraffin embedded; FIGO, International Federation of Gynecology and Obstetrics; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; *HRAS*, Harvey rat sarcoma virus; HUS, Haukeland University Hospital; IHC, immunohistochemistry; ISH, in situ hybridization; *NOTCH*, neurogenic locus notch homolog protein; PCR, polymerase chain reaction; PFS, progression-free survival; REK, Regional Committee for Medical and Health Research Ethics; TMA, tissue microarray; VSCC, vulva squamous cell carcinoma.

significantly with increased progression-free survival ($p = 0.040$) and DSS ($p = 0.045$), after adjustment for other confounders.

Conclusions: p53 and p16^{INK4A} immunohistochemistry stratify VSCC cohort into three subtypes with *TP53*mutated patients having the worst prognosis. The detection of hrHPV mRNA by ISH was an independent predictor of increased survival. Thus, the combined detection of p53 and HPV mRNA might improve risk stratification in VSCC.

KEYWORDS
human papilloma virus, in situ hybridization, prognosis, *TP53*, vulva cancer, vulva squamous cell carcinoma

1 | INTRODUCTION

Vulva squamous cell carcinoma (VSCC) is a rare, heterogeneous disease that has been increasing in incidence, particularly in women below 60 years.[1,23,4] Surgery, along with radiochemotherapy, have been the cornerstone in VSCC treatment for several decades.[5] However, currently, there is no effective treatment for locally advanced radioresistant disease or systemic disease.[6] The recurrence rates are high at 12%–37%, and the five-year survival rate is only 25%–50% in patients with recurrent/metastatic disease.[5,7] While the pathogenesis of VSCC has been extensively studied, molecular risk stratification and phenotypic profiling have only recently been applied in clinical trials.

Two distinct etiopathogenic pathways of VSCC have been elucidated: VSCC with human papilloma virus infection (HPV-associated) and VSCC with chronic dermatitis, such as lichen sclerosus, without HPV (HPV-independent).[1] HPV-associated VSCC is often observed in younger women and accounts for 20%–35% of VSCC cases.[6] HPV-associated VSCC has a mostly basaloid and/or warty histology; displays strong, diffused cytoplasmic and nuclear expression ("block" type) of the cell cycle protein p16^{INK4A}; and high expression of Ki67 (a marker of active cell proliferation).[6] HPV-independent VSCC is more common in elderly women and exhibits keratinizing histological profile with atypia, *TP53* mutations, and little to no p16^{INK4A} expression.[6,8] Increase in p53 expression in tumor tissues is considered as an indicator of poor prognosis of VSCC.[9–11] More recently, p16^{INK4A} and p53 immunohistochemistry has been used to define three distinct VSCC subtypes, namely, p16+, p16−/p53wt, and p16−/p53mut, which exhibit significant survival differences.[12]

Patients with HPV-associated carcinomas of the head and neck regions have better survival rates, due to better response to radiotherapy.[13,14] However, the prognostic significance of HPV in VSCC is unclear, as HPV-associated and HPV-independent cancers often have overlapping clinical characteristics.[15–18] With the emergence of subtype-specific treatment algorithms, identification of the HPV status in VSCC has become paramount in clinical practice.[12,19] The World Health Organization 2020 guidelines recommend p16^{INK4A} block-type immunoreactivity and/or positive molecular testing for HPV, combined with p53 testing, for determining the HPV status of VSCC.[20] However, currently, there is no consensus on the ideal HPV detection method, with HPV DNA PCR and p16^{INK4A} immunohistochemistry (IHC) being widely used. Recently, HPV mRNA in situ hybridization (ISH) has been used to obtain precise spatial and quantitative information about transcriptionally active viral HPV in tumor cells. mRNA ISH can be performed on formalin-fixed paraffin-embedded (FFPE) slides and can, thus, be performed routinely.[21,22]

Key message

Individual risk assessment is needed in order to shift to more personalized treatment guidance of women with vulva squamous cell carcinoma. Our results demonstrate that patients positive for HPV mRNA have better clinical outcome, and patients with p53-mutations have the highest risk for recurrence and death. Combining HPV mRNA in-situ hybridization with p53 immunohistochemistry could improve risk stratification.

The primary aim of our study was to determine the *TP53* mutation and p16^{INK4A} status by performing IHC on archival FFPE VSCC tissue samples and to validate molecular subclassification based on this approach. Furthermore, the secondary aim was to compare different HPV detection methods and to investigate associations of clinical, pathological, and immunohistochemical parameters and survival outcomes.

2 | MATERIAL AND METHODS

2.1 | Patient samples and histological evaluation

This retrospective study included 123 patients older than 18 years of age with a primary diagnosis of VSCC between 1999 and 2017 at the Department of Gynecology and Obstetrics, Haukeland University Hospital (HUS), Bergen, Norway. All patients were treatment naïve, and most were included in Bergen Gynecologic Cancer Biobank (GYNCAN). Written informed consent was obtained before enrollment. A few samples were retrieved from the diagnostic biobank at the Department of Pathology, HUS. Patients with neoadjuvant treatment, missing and/or inadequate tissue blocks, and without written

consent were excluded from the study ($n=15$). Clinicopathological information was collected from hospital records, and the study followed the REMARK criteria.[23] Information on age at diagnosis, treatment, recurrences, and disease characteristics (such as the presence of metastasis and International Federation of Gynecology and Obstetrics [FIGO] stage) were obtained from the medical records. Progression-free-survival (PFS, defined as the time interval in months between the date of termination of primary treatment and the date of recurrence or death) and disease-specific survival (DSS, defined as time interval in months from the day of primary treatment to last follow-up or death from disease, with patients alive at last contact or dead from another cause were censored) were obtained from the medical records.

Histopathological parameters such as the worst pattern of invasion, tumor budding, and the pattern of invasion were evaluated jointly by experienced pathologists on hematoxylin and eosin (HE) stained FFPE sections. Worst pattern of invasion was scored as defined in earlier studies.[24] Tumor budding was defined as a single cell or a group of less than five tumor cells present at the worst pattern of invasion of the tumor. The number of tumor buds was evaluated at 20× magnification field in a tissue area within 100 μm of the invasive tumor front.

2.2 | Tissue microarray construction

Due to limited availability of tissue from metastases and recurrences, two 1-mm core samples were taken to construct a tissue microarray (TMA) (one from the tumor center and one from the invading tumor front).

2.3 | IHC and HPV RNA ISH

Whole FFPE tissue sections of primary tumors ($n=123$) were immunostained for p53, Ki67, and $p16^{INK4A}$. For metastases ($n=37$), 74 TMA cores were stained whereas for recurrent VSCC tumors ($n=44$, first-recurrence [$n=35$] and second-recurrence [$n=9$]) 88 TMA cores were stained. Immunohistochemical staining was performed using the BrightVision Ultimate plus goat anti-mouse/rabbit HRP-DAB kit (Medac Diagnostics) according to the manufacturer's instructions. The primary antibodies used were anti-$p16^{INK4A}$, anti-p53 and anti-Ki67. Further information about antibody dilutions and digital quantification can be found in Appendix S1.

Transcriptionally active HPV infection was detected using RNA ISH on TMA sections following the instructions of the RNAscope 2.5 High-Definition Brown Assay kit (Advanced Cell Diagnostics [ACD Biosciences]). Detailed protocol is mentioned in Appendix S1. The whole cohort of 123 patients underwent RNA ISH detection for two probes (a) HPV 16/18 E6 E7 mRNA (cat no. 311121, ACD Biosciences), and (b) HR7 HPV (detects HPV 16, 18, 31, 33, 35, 52, and 58, E6/E7 mRNA; cat no. 312351, ACD Biosciences). Quantification of brown stained HPV mRNA + cells was done by the pathologists using a light microscope and the results were graded as positive or negative for HPV RNA ISH.

2.4 | HPV DNA detection and subtyping by GP5+/6+ consensus primers

HPV DNA detection was performed using a protocol published elsewhere.[25] Briefly, three to five FFPE sections of 10-μm thickness were collected in microcentrifuge tubes, deparaffinized using deparaffinization solution (Qiagen), and digested overnight in ATL buffer and Proteinase K (Qiagen) at 56°C. DNA was then extracted using the E.Z.N.A. tissue DNA Kit (Omega BioTek), and quantified using the Qubit dsDNA BR assay kit (Thermo Fisher Scientific). The extracted DNA was then added to a PCR reaction mastermix together with standard Gp5+/Gp6+ primers and subtyping of HPV positive samples was performed by DNA sequencing (see Appendix S1).

2.5 | Statistical analyses

Correlations between clinicopathological parameters and biomarkers were analyzed using Pearson chi-square test. Log-rank test and Kaplan–Meier curves were used to compare survival outcomes. The Cox regression model was used to investigate the association of each parameter with PFS and DSS. Parameters that exhibited significant risk difference in the univariable analysis were tested for significance in a multivariable analysis. In the multivariable analysis, PFS was adjusted to age and tumor stage and DSS was adjusted to age. The results were summarized in a forest plot as hazard ratios with 95% confidence intervals, and p-values ≤0.05 were considered statistically significant. The sensitivity and specificity of HPV detection methods were plotted against HPV DNA status on a receiver operating curve (ROC). All statistical analyses were performed using SPSS version 26 (IBM Corp.), and forest plots were generated using GraphPad Prism 9.

2.6 | Ethics statement

The study was approved by the Regional Committee for Medical and Health Research Ethics, Norway (REK, REK2017/279) on April 7, 2017. The use of samples from both GYNCAN biobank and the diagnostic biobank (HUS) were also approved by REK (REK West: REK 2014/1907).

3 | RESULTS

3.1 | VSCC subtypes based on $p16^{INK4A}$ and p53 status

The median age at primary diagnosis was 71 years and median follow-up period was 5 years. Most patients (68.3%) had early-stage disease (FIGO stage I and II) and primary treatment was surgery with 80.5% patients undergoing local excision, while the rest of the

TABLE 1 Histological and clinical parameters of the VSCC cohort based on p53 and p16 IHC staining.

Parameters	Total ($n=123$)	*TP53*mut/p16− ($n=55$)	*TP53*wt/p16+ ($n=35$)	*TP53*wt/p16− ($n=33$)	*p*-value
Age at diagnosis					
Years, median (range)	71 (32–102)	76 (32–101)	58 (39–91)	78 (42–102)	0.390
BMI					
Normal	33 (26.9%)	10 (18.2%)	13 (37.1%)	10 (30.3%)	**0.049**
Overweight and obese	56 (45.5%)	32 (58.2%)	14 (40%)	10 (30.3%)	
Unknown	34 (27.6%)	13 (23.6%)	08 (22.9%)	13 (39.4%)	
Smoking					
No	65 (52.8)	33 (60%)	13 (37.1%)	19 (57.6%)	
Yes	26 (21.2)	06 (10.9%)	15[a] (42.9%)	05 (15.2%)	
Unknown	32 (26)	16 (29.1%)	07 (20%)	09 (27.3%)	**0.002**
FIGO stage					
Stage I	64 (52%)	26 (47.3%)	16 (45.7%)	22 (66.7%)	
Stage II	20 (16.3%)	07 (12.7%)	09 (25.7%)	04 (12.1%)	
Stage III	37 (30.1%)	22 (40%)	09 (25.7%)	06 (18.2%)	0.135
Stage IV	02 (1.6%)	00 (0.0%)	01 (2.8%)	01 (3%)	
Tumor size					
≤4 cm	87 (70.7%)	37 (67.3%)	27 (77.1%)	23 (69.7%)	0.598
>4 cm	36 (29.3%)	18 (32.7%)	08 (22.9%)	10 (30.3%)	
Lymphadenectomy					
No	32 (26%)	12 (21.8%)	11 (31.4%)	09 (27.3%)	
Yes, bilateral	70 (56.9%)	32 (58.2%)	22 (62.8%)	16 (48.5%)	
Yes, regional	21 (17.1%)	11 (20%)	02 (3.6%)	08 (24.2%)	0.535
Lymph node metastasis					
No	85 (69.1%)	31 (56.4%)	28 (80%)	26 (78.8%)	
Yes	38 (30.9%)	24 (43.6%)	07 (20%)	07 (21.2%)	**0.023**
Recurrence of cancer					
No	82 (66.7%)	31 (56.4%)	28 (80%)	23 (69.7%)	
Yes	41 (33.3%)	24 (43.6%)	07 (20%)	10 (30.3%)	
If yes					
Localized	30 (73.2%)	20 (83.3%)	03 (42.8%)	07 (70%)	
Regional	08 (19.5%)	04 (16.7%)	02 (28.6%)	02 (20%)	
Distant	03 (7.3%)	00	02 (28.6%)	01 (10%)	0.062
Histological profile					
Basaloid and/or warty	55 (44.7%)	14 (25.5%)	29[a] (82.9%)	12 (36.4%)	
Keratinizing	54 (43.9%)	33 (60%)	03[a] (8.6%)	18 (54.5%)	
Mixed	14 (11.4%)	08 (14.5%)	03 (8.6%)	03 (9.1%)	**<0.001**
HPV DNA					
No	87 (70.7%)	54 (98.2%)	02[a] (5.7%)	31 (93.9%)	
Yes	36 (29.3%)	01 (1.8%)	33[a] (94.3%)	02 (6.1%)	**<0.001**
High-risk HPV mRNA ISH					
No	92 (74.8%)	55 (100%)	05[a] (14.3%)	32 (97%)	
Yes	31 (25.2%)	00	30[a] (85.7%)	01 (3%)	**<0.001**
HPV 16/18 mRNA ISH					
No	94 (76.4%)	55 (100%)	08[a] (22.9%)	31 (93.9%)	
Yes	29 (23.6%)	00	27[a] (77.1%)	02 (6.1%)	**<0.001**

TABLE 1 (Continued)

Parameters	Total ($n=123$)	*TP53*mut/p16− ($n=55$)	*TP53*wt/p16+ ($n=35$)	*TP53*wt/p16− ($n=33$)	*p*-value
Tumor budding					
<5 buds	74 (60.2%)	27 (49.1%)	28 (80%)	19 (57.6%)	
≥5 buds	49 (39.8%)	28 (50.9%)	07[a] (20%)	14 (42.4%)	**0.013**
Type of invasion					
Cohesive	74 (60.2%)	29 (52.7%)	29[a] (82.9%)	16 (48.5%)	
Trail	49 (39.8%)	26 (47.3%)	06[a] (17.1%)	17 (51.5%)	**0.005**
Worst pattern of invasion					
Type I–III	31 (25.2%)	11 (20%)	11 (31.4%)	09 (27.3%)	
Type IV–V	92 (74.8%)	44 (80%)	24 (68.6%)	24 (72.3%)	0.453

Note: Bold values indicate significance.

Abbreviations: BMI, body mass index; FIGO, The International Federation of Gynecology and Obstetrics; HPV, human papilloma virus; IHC, immunohistochemistry; ISH, in situ hybridization; mut, mutated; VSCC, vulva squamous cell carcinoma; wt, wild-type.

[a]Values indicate a significant contribution of the cell to *p*-value.

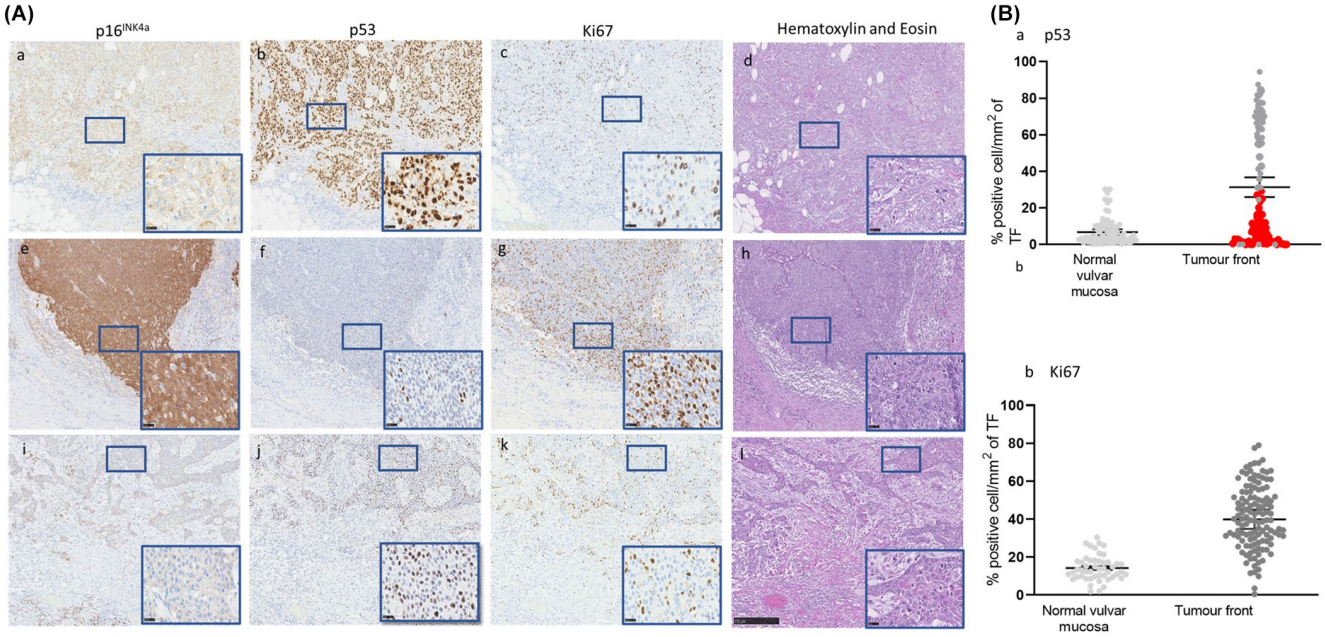

FIGURE 1 Representative immunohistochemical staining of $p16^{INK4A}$, p53, and Ki67. (A) Differences in immunohistochemistry (IHC) staining of $p16^{INK4A}$ (a, e, i), p53 (b, f, j), and Ki67 (c, g, k) as detected by diaminobenzidine staining (DAB, brown color) in vulva squamous cell carcinoma samples. Representative hematoxylin and eosin staining of the same area is shown in d, h, and l (scale bar = 250 μm). The inset sections show higher magnification images of the corresponding boxed areas (scale bar = 50 μm). (B) Digital quantification of IHC staining of (A) p53 and (B) Ki67. The $p16^{INK4A}$ positive tumors are shown as red dots in the digital quantification of p53 staining. Data are presented as median with 95% confidence interval value of the percentage of cells that were positive per mm^2 of the tumor front (TF).

patients (19.5%) had hemi-vulvectomy. Lymphadenectomy was performed in 91/123 patients (74.0%) as part of their primary treatment and in 38 patients, lymph node metastasis was identified. In 33/38 (86.5%) cases, extra nodal extensions were observed. The clinicopathological characteristics of the cohort are provided in Table 1.

A total of 35 primary tumors (35/123, 29%) demonstrated strong cytoplasmic and nuclear expression ("block" type) of $p16^{INK4A}$ (Figure 1A a, e, & i) and were considered positive. Most primary tumors (82.9%) had basaloid and/or warty features (Figure 1A d, h, & l). p53 was expressed predominantly in the nucleus of cancer cells (Figure 1A b, f, & j). Based on IHC staining patterns previously described for identification of *TP53* mutations in VSCC,[12] 55 primary tumors (44.4%) were considered to be *TP53* mutated, and all of them were negative for $p16^{INK4A}$ (Figures 1B a, 2). Most of these tumors (60%) were of the keratinizing type, and 14.5% were of the mixed type exhibiting both basaloid and

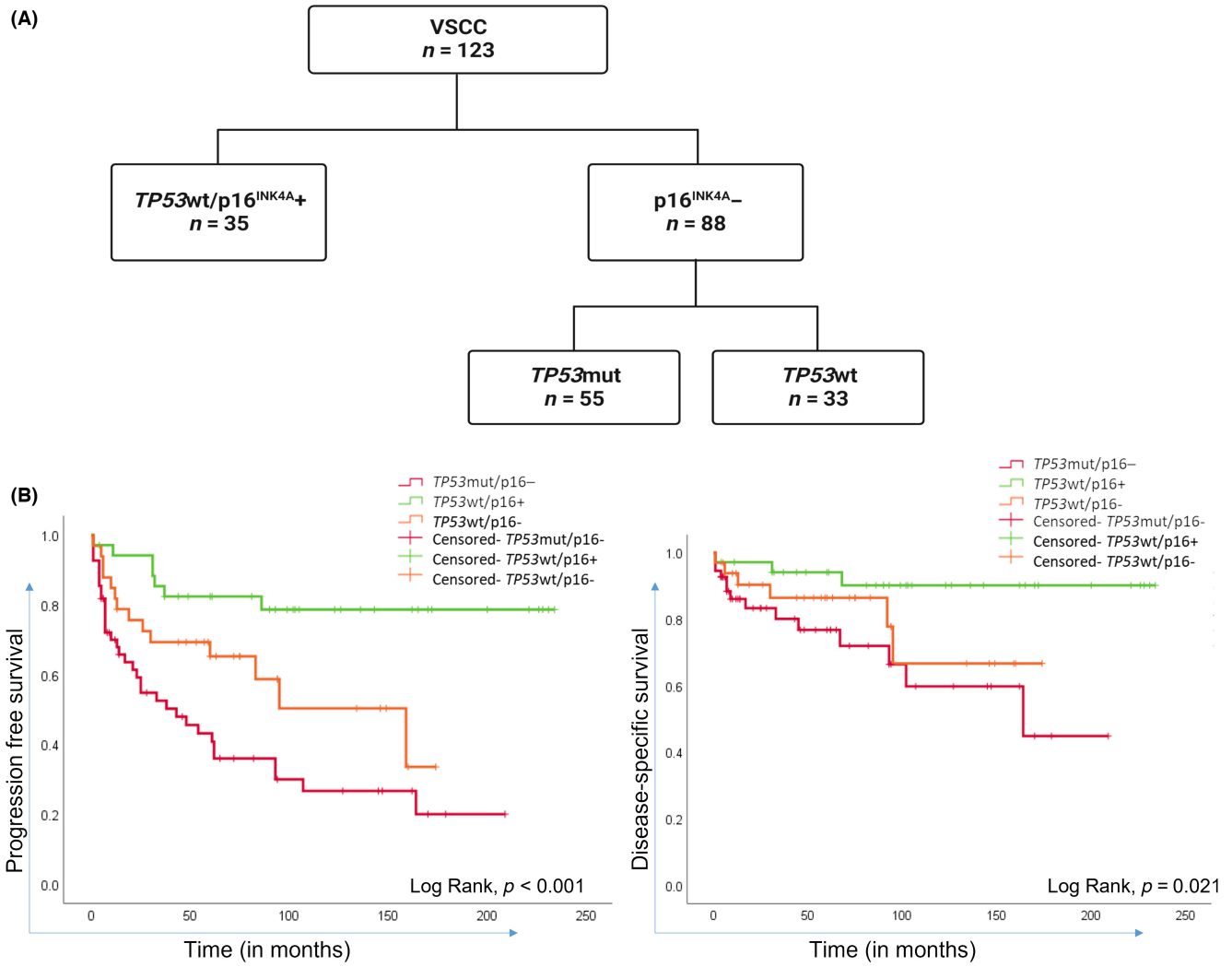

FIGURE 2 Vulva squamous cell carcinoma subtypes based on p16^{INK4A} and *TP53* status. (A) Cases segregated by p16^{INK4A} and *TP53* status (+ indicates positive cases, − indicates negative cases). (B) Kaplan–Meier curves for progression-free and disease-specific survival of different subtypes based on p16^{INK4A} and *TP53* status.

keratinizing type histology. Ki67 was present in the nucleus of cancer cells (Figure 1A c, g, & k) with 50 primary tumors (40.3%) being positive for Ki67. The percentage of Ki67-positive cells at the invasive tumor front was significantly higher than that of the juxta-tumor normal epithelial (Figure 1B b, $p < 0.001$). Several tumors (33/123, 26.8%) were *TP53*wt and p16− and did not fall into any of the classical subtypes of VSCC (Figure 2A; Table 1). Of note, there were no cases with p16+ and *TP53* mutation as reported in previous studies.[12] Compared to the two classically described groups, patients with TP53wt/p16− tumors were significantly older at diagnosis (75.6 vs. 71.6 years for TP53mut/p16−, 61.3 years for TP53wt/p16+ tumors; $p = 0.001$). Moreover, they showed similar rates of lymph-node metastases to TP53wt/p16+ tumors (21.2% vs 20%, respectively), but differed significantly to the TP53mut/p16− tumors (21.2% vs 43.6%; $p = 0.023$). Pathological features such as tumor budding and type of invasion were significantly different in TP53wt/p16− tumors as compared to TP53wt/p16+ but comparable to TP53mut/p16− (Table 1). Since the FIGO stages at diagnosis of these groups did not significantly differ, survival status were compared. 47.3% of patients with *TP53*wt/p16− tumors demonstrated disease progression compared to 63.6% in the *TP53*mut/p16− group and 15.4% in the *TP53*wt/p16+ group. Similarly, 80.8% patients in the *TP53*wt/p16− subgroup were alive (based on disease-specific survival) as compared to 74.5% in the *TP53*mut/p16− group and 92.3% in the *TP53*wt/p16+ group during the study period. Kaplan–Meier analysis revealed that patients with *TP53*wt/p16+ tumors had better PFS and DSS (log-rank test: $p < 0.001$ and $p = 0.021$, respectively) than patients with *TP53*mut/p16− and *TP53*wt/p16− tumors (Figure 2B).

3.2 | *TP53* mutation status as an independent prognostic factor of worse PFS

TP53 mutations were detected in 17 metastases (45.9%), 10 first recurrences (28.6%), and one second recurrence (11.1%) (Table S1).

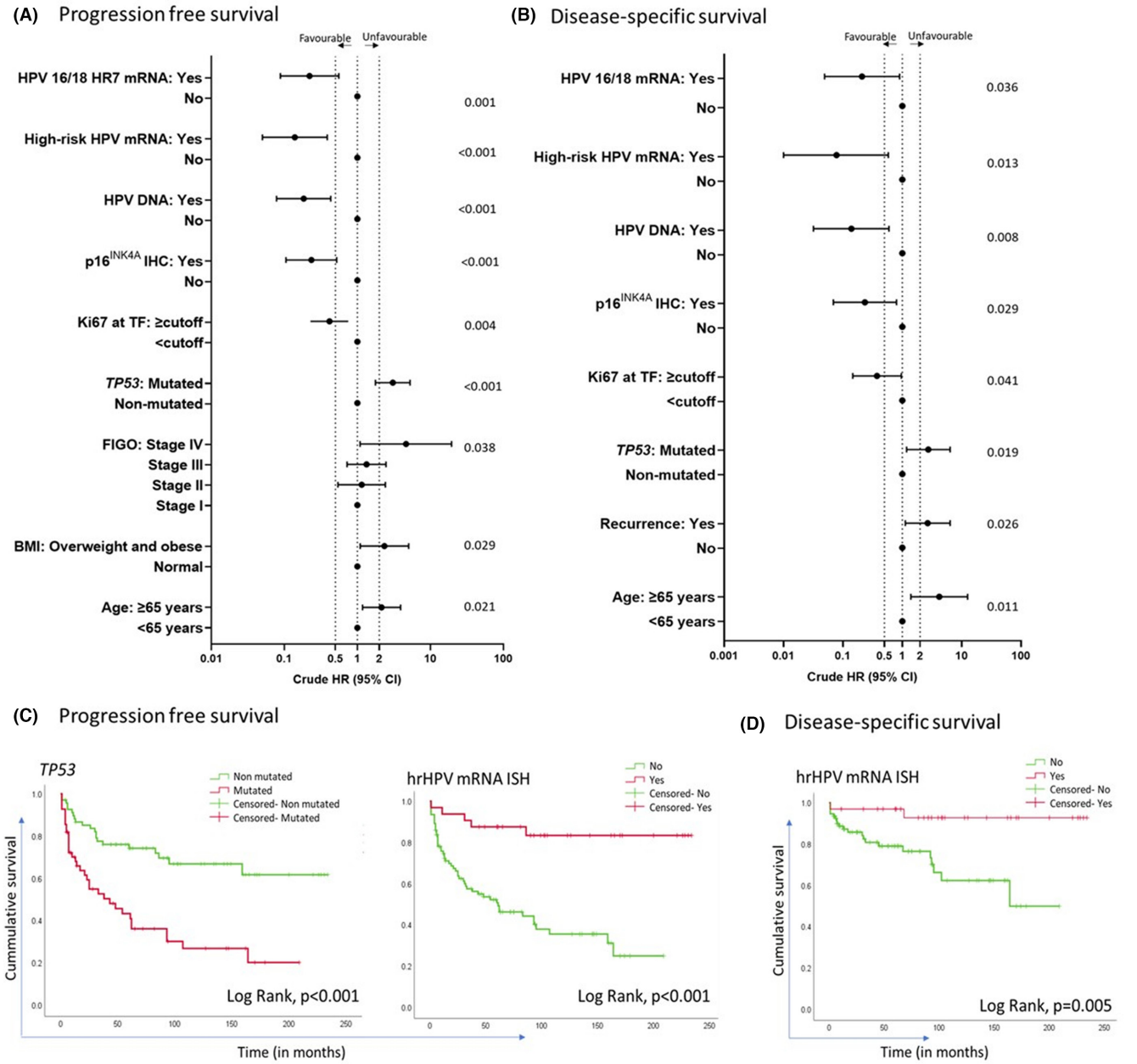

FIGURE 3 Forest box plots depicting the association of various parameters with survival. The hazard ratios (HR) with 95% confidence interval and *p*-values are shown for the association of different parameters with (A) progression-free survival and (B) disease-specific survival. Kaplan–Meier survival curves for parameters that were independent prognostic indicators according to multivariable Cox regression analysis. (C) Progression-free survival of the *TP53*-mutated and hrHPV mRNA ISH subgroups, and (D) disease-specific survival of the hrHPV mRNA ISH subgroup.

Six of the metastases (16.2%) showed differences in the p53 staining profile: *TP53* mutation was present in the primary tumor but absent in the corresponding recurrent tumor, or vice versa. In most cases (4/6, 66.7%), the recurrent tumor had gained p53 expression. Further, 27 out of the 35 (77.4%) first recurrences had similar p53 profiles as their primary tumor. Seven (7/8, 87.5%) tumors had a *TP53* mutation in the primary tumor but was absent in the recurrent tumor with a representative sample shown (Figure S1a, d, & g). No difference in $p16^{INK4A}$ staining or histological features (that is, keratinizing type vs. nonkeratinizing type) was observed between samples from the same patient (Figures S1b, e, & h and S1c, f, & i). Univariable Cox regression analysis revealed that the presence of *TP53* mutation was significantly associated with PFS ($p < 0.001$) and DSS ($p = 0.019$) (Figure 3A,B). The *TP53* mutation status remained an independent prognostic predictor of PFS ($p = 0.024$) after adjustment for FIGO stage in a multivariable Cox regression model (Table 2).

TABLE 2 Results of multivariable analysis of the significant variables associated with progression-free survival and disease-specific survival.

Parameters	Hazard ratio	95% CI	*p*-value
Progression-free survival[a]			
TP53 (mutated)	2.11	1.11–4.02	**0.024**
Ki67 (≥cutoff)	0.64	0.34–1.19	0.158
$p16^{INK4A}$ IHC (yes)	2.03	0.47–8.75	0.342
HPV DNA (yes)	0.66	0.07–6.29	0.718
HPV 16/18 mRNA ISH (yes)	1.31	0.13–13.73	0.822
hrHPV mRNA ISH (yes)	0.13	0.02–0.91	**0.040**
Disease-specific survival[b]			
Ki67 (≥cutoff)	0.52	0.19–1.44	0.207
TP53 (mutated)	1.82	0.66–5.00	0.246
HPV DNA (yes)	0.93	0.07–11.82	0.953
$p16^{INK4A}$ IHC (yes)	3.50	0.45–27.42	0.234
hrHPV mRNA ISH (yes)	0.04	0.02–0.94	**0.045**

Note: Significant values are presented in bold.

[a]Adjusted for tumor stage.

[b]Adjusted for tumor stage and age.

Abbreviations: CI, confidence interval; HPV, human papilloma virus infection; IHC, immunohistochemistry; mRNA ISH, mRNA in situ hybridization.

3.3 | Comparison of the specificity and sensitivity of different HPV detection methods

HPV DNA was detected in 36 (29.3%) patients (30 for HPV 16/18 and 6 for HPV 33). According to ISH, HPV 16/18 mRNA and hrHPV mRNA were detected in 29 (23.6%) and 31 (25.2%) patients, respectively. All the cases that were positive for HPV mRNA by ISH ($n=35$, combined for hrHPV and HPV 16/18 mRNA cases) were positive for HPV DNA by PCR (Figure 4A). Compared to HPV DNA PCR, the area under the curve (AUC) for HPV 16/18 mRNA ISH was 0.903 (95% CI, 0.82–0.98); hrHPV mRNA ISH, 0.931 (95% CI, 0.86–0.99); and $p16^{INK4A}$ IHC, 0.947 (95% CI, 0.89–1.00) (Figure 4B; Table S2). Out of 88 HPV mRNA ISH- cases, two were $p16^{INK4A}$ IHC+ (both negative for HPV DNA) with a representative image shown (Figure 4C a–c). Interestingly, both these discordant cases were p53wt (Figure S2). On the other hand, one case that was negative for $p16^{INK4A}$ was positive for HPV DNA, HPV 16/18 mRNA, and hrHPV mRNA (Figure 4C e–g). Both cases were p53wt (Figure 4C d and h).

3.4 | hrHPV mRNA as an independent prognostic factor for improved DSS

In the univariable analysis, age (>65 years), higher FIGO stage and body mass index were associated with decreased PFS, whereas $p16^{INK4A}$, HPV DNA, hrHPV mRNA, and HPV 16/18 mRNA were significantly associated with increased PFS (Figure S3). Similarly, age > 65 years and recurrence were significantly associated with poor DSS. Ki67, $p16^{INK4A}$, HPV DNA, hrHPV mRNA, and HPV 16/18 mRNA were significantly associated with improved DSS (Figure S4). In the multivariable Cox regression analysis, only hrHPV mRNA status ($p=0.040$) was an independent prognostic indicator of PFS when adjusted for tumor stage (Table 2; Figure 3C). For DSS, only hrHPV mRNA status showed a significant association with better DSS after adjustment for tumor stage and age ($p=0.045$) (Table 2; Figure 3D). Since hrHPV mRNA status was an independent prognostic predictor of improved PFS and DSS, the correlation of all the clinicopathological parameters was examined using the Pearson chi-square test (Table S3).

4 | DISCUSSION

Recent decades have seen some improvements in VSCC therapeutic options.[1,12] Moreover, advances in molecular classification have led to suggestions that HPV-associated tumors are less aggressive and have better prognosis.[19,26] Despite this, the treatment regimen is similar for HPV-associated and HPV-independent VSCC.[8] To be able to shift towards personalized medicine, individual risk for VSCC progression needs to be assessed upfront on biopsy tissue or other biological material. In this regard, the questions addressed in this study were, first to determine if distinct molecular subtypes defined by *TP53* mutation and $p16^{INK4A}$ status are of prognostic significance in VSCC and second to identify the most feasible method to detect active HPV infection. Data from this study suggests that mutated *TP53* status is an independent prognostic factor of decreased PFS ($p=0.024$) after adjusting for FIGO stage. Furthermore, different HPV detection methods have excellent concordance between them in detecting HPV. hrHPV mRNA ISH correlated significantly with increased PFS ($p=0.040$) and DSS ($p=0.045$) after adjusting for other confounders.

HPV-independent VSCC is frequently associated with *TP53* mutations.[6,12,27] In the present cohort, *TP53* mutation significantly correlated with shorter PFS in a multivariable Cox regression model. This data corroborates with an earlier study on VSCC by Dong et al. ($n=97$).[9] Further, recent studies have reported the presence of a clinically relevant *TP53*wt/p16− subgroup in addition to the classical *TP53*mut/p16− and *TP53*wt/p16+ subgroups in VSCC.[12,27] In this study, 26.8% of the VSCCs could be classified under this newly described *TP53*wt/p16− subgroup. Although the molecular mechanism of oncogenesis is unclear in this group, several molecular markers have been proposed to influence clinical outcomes,[27] including *EGFR*, *NOTCH1*, and *HRAS*.[28] Recent studies have identified frequent mutations in *NOTCH1* and *HRAS* in p53−/p16− subgroups in both VSCC[5,12,28] and Head and Neck SCC.[29,30] Additionally, studies have shown that *TP53*wt/p16− tumors have intermediate recurrence rates that are more similar to those of the *TP53*mut/p16− subgroup than the TP53wt/p16+ subgroup.[12] Our results on the PFS and DSS of the three subtypes are in agreement with these previous studies. The present study also looked at p53

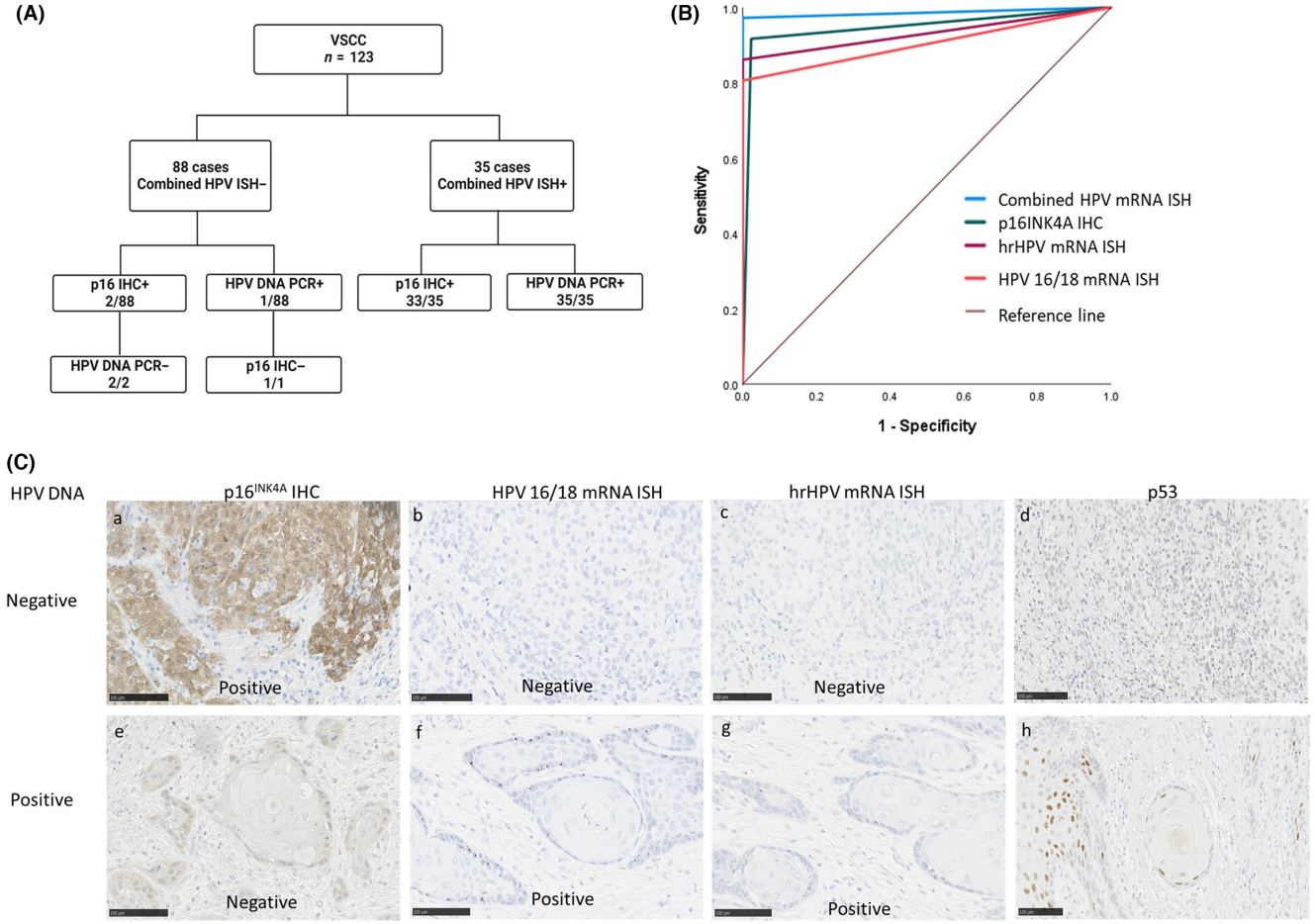

FIGURE 4 Comparison of the different human papilloma virus infection (HPV) detection methods. (A) Decision tree for comparison of HPV status. HPV DNA PCR, p16^{INK4A} IHC, HPV 16/18, and high-risk HPV mRNA ISH was performed on all tissue samples with the tumors initially divided into the combined HPV ISH- and ISH+ groups. Later, based on p16^{INK4A} IHC and HPV DNA PCR, three subgroups were defined. (B) Receiver operator characteristic (ROC) curves were used to assess the discriminatory accuracy of p16^{INK4A} IHC, HPV 16/18 mRNA ISH, hrHPV mRNA ISH, and combined HPV 16/18 and hrHPV mRNA ISH against HPV DNA PCR. (C) Cases in which the results of HPV DNA PCR, p16^{INK4A} IHC, and HPV mRNA ISH were not in agreement. Representative images for a p16+ (a), HPV 16/18 mRNA ISH-(b), hrHPV mRNA ISH (c) and p53wt (d) case. Representative images for a p16− (e), HPV DNA+ (f), HPV 16/18 mRNA ISH+ (g), hrHPV mRNA ISH and p53wt (h) case (scale bar = 100 μm).

and p16 status in recurrent and metastatic settings. Most recurrent tumors had similar p53 and p16 staining profile. However, 22.8% recurrent tumors had a different p53 staining pattern as compared to its primary tumor suggesting loss or gain of p53 expression may be due to tumor heterogeneity or "de novo" mutation that tumors acquire during disease progression. Our data corroborates with the those of Lerias et al. who reported similar observations in recurrent settings.[18]

Previous studies investigating the role of HPV (detected by p16^{INK4A} IHC, HPV DNA PCR, or HPV mRNA ISH) in VSCC reported contradictory results.[15–18] This could be attributed to differences in the sensitivity and specificity of different HPV detection methods. Currently, p16^{INK4A} IHC and HPV DNA PCR are the most common methods.[21] In our study, both p16^{INK4A} IHC and HPV DNA PCR showed prognostic significance for the prediction of PFS and DSS only in the univariable Cox regression analysis. However, while p16^{INK4A} immunostaining is easy to carry out and is widely available, it lacks specificity for HPV since it might be upregulated in cancer cells by several other mechanisms independent of HPV.[31] Further, the criteria for defining p16^{INK4A} positivity is based on studies on oropharyngeal and cervical cancers, where HPV infection is common.[31,32] In contrast, in vulva cancers, the presence of HPV infection varies widely and therefore applicability of these criteria for vulva cancers needs to be re-examined.[33] HPV DNA PCR is too sensitive, does not perform as well on archival FFPE samples, and does not provide spatial and transcriptional information.

Detection of HPV mRNA using ISH may help overcome the limitations of these methods, as it allows for direct visualization of transcriptionally active viral HPV load in FFPE sections and may, therefore, be the key to detecting high-risk HPV-type tumors.[21,22] In our study, the specificity and sensitivity of combined mRNA ISH (hrHPV mRNA and HPV 16/18 HPV mRNA) were 100% and 97.2%, respectively. In addition, hrHPV mRNA ISH was the only method for HPV detection that demonstrated a significant association of HPV

infection with increased PFS and DSS, even after adjustment for other confounders.

Of note, there were three discrepant cases of p16 IHC and HPV nucleic acid tests. Two of the three cases were p16 IHC+ and HPV nucleic acid negative. These cases were not *TP53* mutated based on the IHC staining and the histology was of mixed type. Previous studies suggest that these cases are likely false positive p16 cases that are not related to HPV and could be due to other genomic alterations in the retinoblastoma protein pathway.[34] Indeed, such discordant cases have been reported in a recent large multicenter international study of 7895 patients with oropharyngeal cancer with p16 IHC+/HPV-independent discordant cases having significantly worse outcomes than p16 IHC+/HPV-associated true HPV cases.[34] However, the present study is underpowered to validate this in VSCC and further multicenter studies are warranted to confirm this.

Clinical parameters such as age more than 65 years, FIGO stage, and body-mass index (overweight and obese) were unfavorable prognostic factors for PFS whereas only age correlated with DSS, which correspond with earlier findings.[35] A significant association between age and prognosis is not surprising, since several studies also have reported that HPV-associated tumors occur more frequently in younger patients and have better prognosis.[36] No prognostic significance was observed between other parameters and survival as reported in other SCCs.[37] No prognostic value was observed between patients with lymph node metastases larger or equal to 5 mm with inferior survival as reported by Lérias et al.[18] A possible explanation is that in our cohort, 81% of patients with lymph node metastasis also had lymph node metastasis with size larger or equal to 5 mm, and thus the data was underpowered to detect an effect.

The limitations of this study include those inherent to the use of TMAs, affecting the identification of intratumoral heterogeneity, which could be clinically significant. In addition, the specificity and sensitivity of p53 IHC for the detection of *TP53* mutation is not 100%, which might explain absence of p53mut/p16+ subtype in our study. Moreover, due to differences in data presented in the patient journals over the years, data for the selected variables could not be identified for all patients. Finally, owing to the small sample size, the results need to be validated in larger cohorts.

5 | CONCLUSION

The present findings indicate the presence of three distinct VSCC subtypes based on p53 and $p16^{INK4A}$ IHC, with $p16^{INK4A}$+ patients having a better prognosis. In addition, there exists a strong concordance between different HPV detection methods, among which hrHPV mRNA detection by ISH was found to be an independent prognostic predictor of improved survival. We postulate that combining HPV mRNA ISH with p53 IHC could improve risk stratification in VSCC.

AUTHOR CONTRIBUTIONS

HND: methodology, data curation, formal analysis, funding acquisition, writing of the original draft. RE: methodology, data curation, formal analysis. ST and IBMK: methodology, data curation. SF: data curation, review and editing, formal analysis. LCVT and ESN: data curation, review and editing. ACJ: data curation, review and editing, supervision. OKV: conceptualization, writing original draft. DEC: data curation, conceptualization, funding acquisition, writing original draft, supervision. LB: conceptualization, funding acquisition, writing original draft, supervision. All authors read and approved of the final version of this manuscript.

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CONFLICT OF INTEREST STATEMENT

LCVT and LB report institutional funding received from AstraZeneca for an ovarian cancer clinical trial; LCVT received honoraria from Bayer and Astra Zeneca and advisory board/consulting speaker fees from Eisai Co.; LB reports payment for expert testimony on patent application and a leadership role in the Nordic Society of Gynecological Oncology (NSGO) clinical trial unit and oncology forum. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ORCID

Harsh Nitin Dongre https://orcid.org/0000-0003-4378-2869
Line Bjørge https://orcid.org/0000-0002-0240-2770

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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