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GYNECOLOGY

p53 and p16 expression profiles in vulvar cancer: a translational analysis by the Arbeitsgemeinschaft Gynäkologische Onkologie Chemo and Radiotherapy in Epithelial Vulvar Cancer study group

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BACKGROUND: There are 2 known pathways for tumorigenesis of vulvar squamous cell carcinoma—a human papillomavirus—dependent pathway characterized by p16 overexpression and a human papillomavirus—independent pathway linked to lichen sclerosus, characterized by *TP53* mutation. A correlation of human papillomavirus dependency with a favorable prognosis has been proposed.

OBJECTIVE: The objective of the study was to further understand the role of human papillomavirus and p53 status in vulvar squamous cell carcinoma and characterize its clinical relevance.

STUDY DESIGN: The Arbeitsgemeinschaft Gynaecological Oncology Chemo and Radiotherapy in Epithelial Vulvar Cancer-1 study is a retrospective cohort study of 1618 patients with primary vulvar squamous cell carcinoma Fédération Internationale de Gynécologie et d'Obstétrique stage ≥1B treated at 29 gynecologic cancer centers in Germany between 1998 and 2008. For this translational substudy, formalin-fixed paraffin-embedded tissue was collected. A tissue microarray was constructed (n=652 samples); p16 and p53 expression was determined by immunohistochemistry. Human papillomavirus status and subtype were analyzed by polymerase chain reaction.

RESULTS: p16 immunohistochemistry was positive in 166 of 550 tumors (30.2%); p53 staining in 187 of 597 tumors (31.3%). Only tumors with available information regarding p16 and p53 immunohistochemistry

and without p53 silent expression pattern were further analyzed (n=411); 3 groups were defined: p53+ (n=163), p16+/p53− (n=132), and p16−/p53− (n=116). Human papillomavirus DNA was detected in 85.6% of p16+/p53− tumors; human papillomavirus-16 was the most common subtype (86.3%). Patients with p16+ tumors were younger (64 vs 72 years for p53+, respectively, 69 years for p16−/p53− tumors; *P*<.0001) and showed lower rates of lymph-node involvement (28.0% vs 42.3% for p53+, respectively, 30.2% for p16−/p53− tumors; *P*=.050). Notably, 2-year-disease-free and overall survival rates were significantly different among the groups: disease-free survival, 47.1% (p53+), 60.2% (p16−/p53−), and 63.9% (p16+/p53−) (*P*<.001); overall survival, 70.4% (p53+), 75.4% (p16−/p53−), and 82.5% (p16+/p53−) (*P*=.002). In multivariate analysis, the p16+/p53− phenotype showed a consistently improved prognosis compared with the other groups (hazard ratio, 0.66; 95% confidence interval, 0.44−0.99; *P*=.042).

CONCLUSION: p16 overexpression is associated with an improved prognosis whereas p53 positivity is linked to an adverse outcome. Our data support the hypothesis of a clinically relevant third subgroup of vulvar squamous cell carcinoma with a p53−/p16− phenotype showing an intermediate prognosis that needs to be further characterized.

Key words: HPV, prognosis, p16, p53, vulvar cancer

Introduction

Over the last 15 years, the incidence of vulvar squamous cell cancer (VSCC) has almost doubled in Germany; in particular, the proportion of younger females at the age between 30 and 49 years affected by the disease has been increasing.[1,2]

However, most women diagnosed are still at the age of 70 to 75 years, and worldwide, VSCC is regarded as a rare disease.[3] According to currently available evidence, there are 2 major pathways for tumorigenesis of VSCC: a human papillomavirus (HPV) dependent with p16 overexpression as a potential surrogate for HPV-associated transformation and an HPV-independent route linked to lichen sclerosus, characterized by *TP53* mutation.[4−6] The understanding of the further molecular landscape underlying VSCC development is only just evolving.[7−9] HPV association was proposed in 20% to 40% of all VSCC cases with HPV-16 being the most commonly

identified subtype (75% of all HPV-related cases).[10−12] In head and neck cancers, which show a similar biologic behavior compared with vulvar cancer, HPV-related disease has repeatedly been demonstrated to have a much more favorable prognosis and a better response to chemoradiation.[13,14] In contrast, no consistent data with regard to an improved prognosis for patients with high-risk HPV-related disease are available for VSCC.[15−23] Reasons for the ambiguous results of the role and frequency of HPV in VSCC might be different detection methods, small and heterogeneous patient cohorts, and the retrospective character of the available

AJOG at a Glance

Why was this study conducted?
For vulvar squamous cell cancer (VSCC), data regarding a more favorable prognosis for patients with human papillomavirus (HPV)—related tumors have been inconsistent. This study is the translational part of the exceptionally large and clinically well characterized Arbeitsgemeinschaft Gynäkologische Onkologie Chemo and Radiotherapy in Epithelial Vulvar Cancer-1 study of 1618 patients with VSCC that aims to further understand the role of HPV and p53 in VSCC.

Key findings
In this analysis, we show that HPV driveness/p16 overexpression is associated with an improved prognosis in VSCC, whereas p53 overexpression is linked to an adverse outcome with lower 2-year disease-free and overall survival rates.

What does this add to what is known?
Our study supports a clinically relevant third subgroup of tumors showing neither p16 nor p53 overexpression that has an intermediate prognosis.

studies. Information on the prognostic relevance of *TP53* mutation and consecutive p53 expression in VSCC in view of the increasing knowledge on HPV is similarly restricted.[6,24] In preinvasive disease, there is robust evidence of a higher progression rate to VSCC in case of non-HPV-related differentiated vulvar intraepithelial neoplasia (VIN) with p53 overexpression, whereas the usual type VIN lesions show lower progression rates and lesser potential for recurrence.[25] In a systematic review from Sand et al,[26] p53 expression (n=310) was also associated with a poorer prognosis (hazard ratio [HR], 1.81 for overall survival [OS]) in invasive disease. Unfortunately, there are very few and small studies investigating all markers (p16, HPV, p53) in the same patient cohort to allow for a valuation with regard to distribution and prognostic differences.[19,27,28]

Therefore, to further understand the role of HPV and p53 in VSCC, we analyzed tumor samples from the Arbeitsgemeinschaft Gynäkologische Onkologie Chemo and Radiotherapy in Epithelial Vulvar Cancer-1 (AGO-CaRE-1) study, an exceptionally large and clinically well characterized cohort from 29 German cancer centers.[29]

Materials and Methods
The AGO-CaRE translational study is a substudy of the AGO-CaRE-1 study. AGO-CaRE-1 is a large retrospective study, evaluating treatment patterns and prognostic factors in vulvar cancer. Participating institutions included all patients with the diagnosis of invasive vulvar cancer stage >pT1a independent of the mode and initial place of treatment. Detailed information about the recruitment and data collection were published by Mahner et al[29]. In short, 1618 adult patients with stage IB—IV VSCC (Union for International Cancer Control version 6[30]), being treated between 1998 and 2008 at 29 AGO cancer centers in Germany, were included. Patient data collection was performed retrospectively between February and December 2011. Documentation and analysis were done through a specifically designed centralized database by the AGO study group. In the database, tumor characteristics and aspects of surgical and nonsurgical treatment were collected including: tumor, nodes, and metastasis stage, tumor size, depth of invasion, grade, number and localization of lymph nodes involved, surgical therapy of the vulva and nodes, pathologic resection margin, total dosage and fields of irradiation, and, if applicable, agent and dosage of chemotherapy and date and treatment of recurrent disease and/or date of last contact or death. Furthermore, patient characteristics such as Eastern Cooperative Oncology Group (ECOG) performance status

and relevant comorbidities were documented. To account for possible bias from informative missing values, we introduced the category "unknown" for each variable to keep all patients in the analysis.

For this CaRE translational substudy, available formalin-fixed paraffin-embedded (FFPE) tissue from the patients documented in the AGO-CaRE-1 database was collected centrally (n=807).

The AGO-CaRE-1 study and the translational substudy were approved by each local ethics committee (leading vote: Hamburg [reference number PV3658]) and registered with ClinicalTrials.gov (NCT01304667).

DNA isolation and human papillomavirus polymerase chain reaction
From each FFPE tissue block, 3×10 μm thick sections were cut and DNA isolation was performed using the Nucleo-Spin DNA FFPE XS kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. To minimize the potential for polymerase chain reaction (PCR) contamination, the microtome blade was cleaned between each block. In addition, negative control blocks were regularly cut and analyzed in between. DNA quality was first proven by PCR using actin-specific primers. HPV status was analyzed by PCR using GP5+/GP6+ primers as previously described,[31] and each HPV-positive result was validated by genotyping with direct DNA sequencing. p16+, HPV negative cases (n=28) were tested for amplification of human beta globin; if positive (n=20), HPV PCR was repeated with 2 primer sets. GP5+/GP6+ (tttgttactgtggtagatactac/gaaaaataaactgtaaatcatattc) and MY09/MY11 (cgtccaaaaggaaactgagccgtccmarrggawactgagc/gcacagggacataacaatgggcmcagggwcataayaatgg).

Tissue microarray
The tissue microarray (TMA) manufacturing process was described in detail before (PMID:9662379). In brief, 807 vulva study samples were validated by a pathologist with a special focus on gynecologic pathology (E.B.). Because of

too small volume of unequivocal cancer for reliable punching, 155 cases were not usable for TMA construction. From the other 652 samples, 1 tissue core measuring 0.6 mm in diameter was taken for TMA construction resulting in 2 TMA blocks. The presence or absence of tumor tissue was validated by visual inspection of the hematoxylin and eosin–stained TMA slides.

Immunohistochemistry

Freshly cut TMA sections were immunostained at 1 day and in 1 experiment. Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 minutes in an autoclave at pH 7.8 Tris–ethylenediaminetetraacetic acid–citrate buffer. Primary antibody specific for p16 (dilution 1:150, monoclonal antibody, Cat#DIA-P16–OD; dianova GmbH, Hamburg, Germany) and p53 (dilution 1:3600, monoclonal antibody, DO-7; DAKO RTU, Glostrup, Denmark) was applied at 37°C for 60 minutes. Bound antibody was then visualized using the EnVision Kit (DAKO RTU, Glostrup, Denmark) according to the manufacturer's directions. Immunohistochemistry (IHC) expression levels were determined by using a 4-step scoring system: negative, no staining at all; weak, 1+ staining intensity in ≤70% positive tumor cells or 2+ staining intensity in ≤30% positive tumor cells; moderate, 1+ staining intensity in >70% tumor cells, 2+ in >30% but ≤70% positive tumor cells or 3+ in ≤30% positive tumor cells; and strong, 2+ >70% or 3+ >30% positive tumor cells. In the case of p53, only cases in the category "strong" were regarded as positive and therefore *TP53* abnormal. Most *TP53* mutations (>70%) have been described as missense mutations leading to an exceptionally stable p53 protein, resulting in strong p53 expression; the rate of *TP53* knockout mutations resulting in a completely negative expression pattern of p53 varies between 50% and 80% in the currently available sparse literature.[9,32] Furthermore, the proportion of p53 IHC completely negative cases could be overrated owing to array-based evaluation in the current study. Therefore, the mutational status

of those cases remains unclear. Consequently, they were not added to the p53 abnormal group but excluded from further analysis (n=124). For p16, both categories "strong" and "moderate" were considered positive because of the small number of samples in the category "moderate" (n=23) and detection of HPV DNA in the majority of these cases (68%).

Statistical analysis

Analyses were performed using Stata (StataCorp LP, version 14.2; StataCorp LLC, College Station, TX). Quantitative variables were summarized using means and standard deviations, and categorical variables are summarized using absolute and relative frequencies. For the determination of significance, we calculated *P* values using 2-sided tests with a 5% level for significance. Tumor and patient characteristics were compared across groups using analysis of variance (quantitative variables), Pearson's chi-square test, or Fisher's exact test (categorical variables). Disease-free survival (DFS) was defined as the time interval between primary diagnosis and disease progression or death of any cause, and OS was the period resulting from primary diagnosis to death of any cause. Cox regression analyses were conducted to determine prognostic factors in (multivariate) survival analysis. Kaplan-Meier curves were calculated to describe (disease-free) survival in subgroups.

Results
Immunohistochemistry

Of 807 collected tumor samples, 652 had enough tumor volume for being punchable for the TMA and were confirmed as VSCC in central review (University Medical Center Hamburg-Eppendorf). p16 staining was evaluable in 550 TMA spots. Notably, 166 of 550 tumors (30.2%) showed a positive (strong [n=143]/moderate [n=23]) p16 expression, whereas 327 spots were classified as negative and 57 as weak. p53 staining was interpretable in 597 spots; among them, 187 of 597 (31.3%) were classified as positive (strong p53 expression), 174 as negative, 178 as weak, and 58 as moderate (wild-type

pattern) (Figure 1). The combined expression profiles of p53 and p16 of all tumors with available information regarding p16 and p53 IHC staining (n=535) are presented in Table 1. In view of their unclear *TP53* mutational status, we excluded all p16−/p53 completely negative cases (n=105) and the p16+/p53 completely negative cases without HPV detection in the PCR (n=19) from further analyses (Table 2). Only 12 tumors (2.9%) showed a coexpression of p16 and p53. Because *TP53* mutations with strong p53 overexpression is a good explanation for non-HPV–related p16 overexpression, these cases were included in the p53+ group for further analysis. Table 3 shows the tumor and patient characteristics of the 411 cases with regard to the expression subgroups: p53+, p16+/p53−, and p16−/p53−. Interestingly, there was a relevant number of tumors with neither p16 nor p53 overexpression (116 of 411). Compared with the other groups, patients with p16+/p53− tumors were significantly younger at diagnosis (64 vs 72 years for p53+, respectively, 69 years for p16−/p53− tumors; *P*<.0001) and showed lower rates of lymph-node involvement (28.0% vs 42.3% for p53+, respectively, 30.2% for p16−/p53− tumors; *P*=.050) (Table 3). In correspondence with the more advanced age of patients with p53+ tumors, the ECOG performance status of these patients was significantly worse than the other subgroups (ECOG 2, 22.1% vs 15.9% for p16+/p53−, respectively, 15.5% for p16−/p53− tumors; *P*=.009).

Human papillomavirus analyses

HPV DNA was detected via PCR in 204 of 411 included tumors (49.6%); the most common subtype was HPV-16 (176 of 204; 86.2%) (refer to Table 3 for other detected subtypes). With regard to p16 and p53 expression, PCR was positive in 85.6% of the p16+/p53− tumors (113 of 132) and 32.3% of all p16− tumors (Table 3).

Survival analyses

Follow-up was available for 376 of 411 patients. Median duration was 19.3 months (range, 0−208 months).

FIGURE 1
Immunohistochemistry examples

A

HE   HE

p16 strong   p16 negative

B

HE   HE

p53 strong   p53 negative
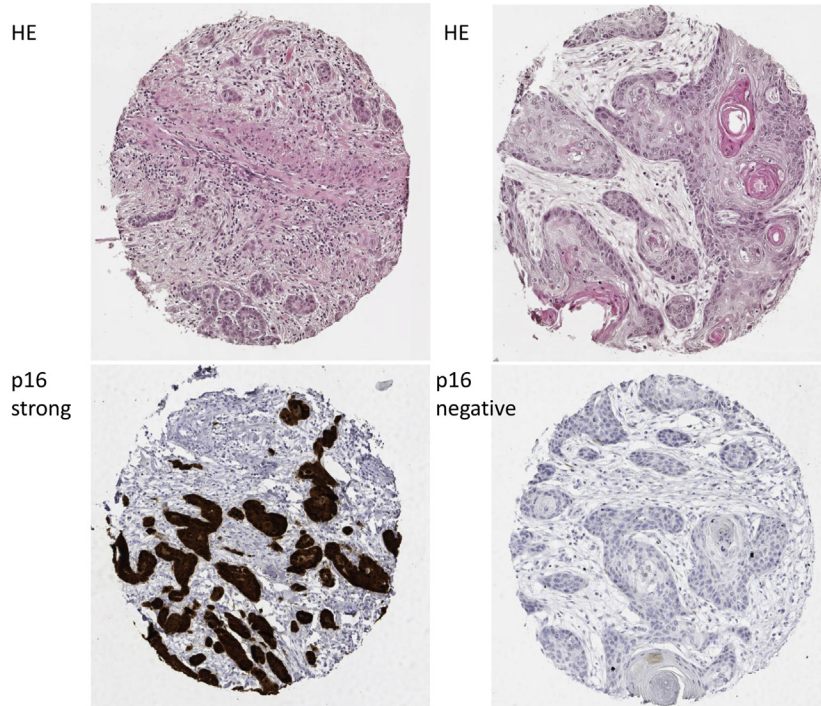
A, p16. **B,** p53.

HE, hematoxylin and eosin.

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Altogether, 122 patients (29.6%) developed disease recurrence (median time to recurrence or death, 32.4 months), 64 of 122 (52.5%) at the vulva only. The localization of recurrent dise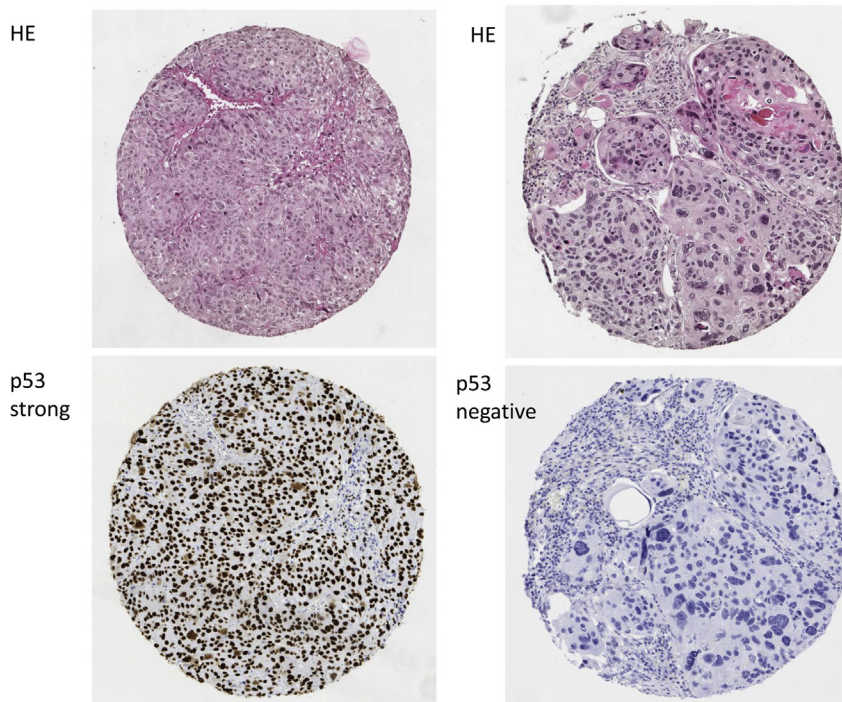ase with regard to the 3 expression subgroups is presented in Table 4. In general, the risk for recurrence was higher in the p53+ (35.0% with recurrent disease) and p16−/p53− (32.0% with recurrent disease) subgroups than the p16+ group (22.7% with disease recurrence). There was no specific recurrence pattern recognizable characterizing the different subgroups.

A total of 102 patients died after a median time of 116.7 months; 2-year DFS and OS rates were significantly different between the groups: DFS, 47.1% (p53+), 60.2% (p16−/p53−), and 63.9% (p16+/p53−) (P<.001); OS, 70.4% (p53+), 75.4% (p16−/p53−), and 82.5% (p16+/p53−) (P=.005), respectively (Figure 2, A and B). The prognostic factors for DFS in multivariate analysis are presented in Table 5. The p16+/p53− phenotype showed a consistently improved prognosis compared with the other groups (HR, 0.66; 95% CI, 0.44−0.99; P=.042).

In the subgroup of p16+/p53− tumors, there was no significant difference in prognosis with regard to HPV detection in PCR (HR, 0.95; 95% CI, 0.841−1.064; P=.353). In the other subgroups, no difference was observed with regard to HPV DNA detection (p16−/p53−, HR, 0.960; 95% CI, 0.870−1.060; p53+, HR, 0.966; 95% CI, 0.867−1.077).

Comment
Principal findings
With the current analysis of the large and well characterized AGO-CaRE-1 cohort, we can now provide very robust evidence that p16 overexpression is associated with an improved prognosis in VSCC, whereas p53 overexpression, respectively, TP53 mutation is linked to an adverse outcome.

Results
Our data confirm the 2 proposed pathways of tumorigenesis in VSCC: an HPV-dependent pathway with p16 overexpression as a surrogate for HPV-

associated transformation and an HPV-independent pathway linked to lichen sclerosus, characterized by TP53 mutation. However, according to our data, there is a further subgroup of VSCC potentially independent from TP53 mutation and HPV. This group (p53− (wild type)/p16− in IHC) was larger than expected in the current cohort (28.2% of the tumors included in the final analysis). In 2017, a TP53 mutation and HPV-independent third subtype of vulvar cancer has firstly been proposed by Nooij and colleagues[8,33] and since then has as well been observed by independent groups on DNA and protein levels. Although the understanding of the molecular characterization of vulvar cancer in depth is still lacking, Nooij et al

refer to a HPV negative/TP53 wild-type subgroup with a high number of NOTCH1 (5 of 10, 50%) and HRAS (5 of 10, 50%) mutations in their targeted NGS analysis of 17 genes in 36 VSCCs. NOTCH1 is a transmembrane receptor involved in differentiation, proliferation, apoptosis, and squamous cell differentiation. HRAS is involved in the prooncogenic PI3K/AKT/mTOR pathway, which has mostly been described as aberrantly activated in HPV-positive VSCC before.[9,22,34,35] In a recent whole-exome NGS analysis by our own group, TP53 mutations and presence of HPV16 with integration of viral E7 gene were mutually exclusive in 34 VSCC samples. In addition, a small "double negative" subgroup of 3 VSCC samples

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TABLE 1
Expression of p53 and p16 (n = 535) in all samples with interpretable spots for both p16 and p53

| p16 | p53 | | | | |
	Negative	Weak	Moderate	Strong	Total
Negative	84	69	32	130	315
Weak	21	11	4	21	57
Moderate	9	4	4	6	23 pos
Strong	45	76	13	6	140 pos
Total	159	160	53	163 pos	535

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TABLE 2
Expression of p53 and p16 (n = 411) after exclusion of p16 negative/weak/p53 negative cases (n = 105) and p53 negative/p16 positive cases without HPV detection (n = 19) owing to unclear TP53 mutational status

| p16 | p53 | | | | |
	Negative	Weak	Moderate	Strong	Total
Negative	0	69	32	130	231
Weak	0	11	4	21	36
Moderate	6	4	4	6	20 pos
Strong	29	76	13	6	124 pos
Total	35	160	53	163 pos	411

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TABLE 3
Patient and tumor characteristics (n=411)

	Total (n=411) n	p16+/p53− (n=132) n	%	p53+ (n=163) n	%	p16−/p53− (n=116) n	%	P value
Age, y (mean, SD)	68.3±14.0	63.5±16.1		71.6±12.1		68.9±12.3		<.0001[a]
Tumor stage[b] pT1b	116	44	33.3	39	23.9	33	28.4	.154[c]
pT2	202	56	42.4	90	55.2	56	48.3	
pT3/4	51	22	16.7	15	9.2	14	12.1	
Unknown	42	10	7.6	19	11.7	13	11.2	
Nodal status pN0	235	86	65.2	80	49.1	69	59.5	.050[c]
pN1	141	37	28.0	69	42.3	35	30.2	
Unknown	35	9	6.8	14	8.6	12	10.3	
hrHPV negative	154	6	4.6	94	57.7	55	47.4	<.001[d]
Unknown[e]	53	13	9.8	23	14.1	17	14.7	
Positive	204	113	85.6	46	28.2	44	37.9	
Type 16	176	101	76.5	37	22.7	38	32.7	
Type 33	15	10	7.6	2	1.2	3	2.6	
Type 18	6	0	0.0	4	2.4	2	1.7	
Type 45	4	1	0.8	2	1.2	1	0.86	
Other	2	1	0.8	1	0.6	0	0.0	
Tumor diameter, mm (mean, SD), n=423	36.3±30.1	39.2±41.5		34.8±21.8		35.3±24.7		.512[a]
Depth of invasion, mm (mean, SD), n=312	8.5±8.7	6.9±5.6		9.7±10.8		8.4±8.2		.135[a]
Grading G1	42	8	6.1	16	9.8	18	15.5	.121[c]
G2	232	79	59.8	95	58.3	58	50.0	
G3	95	35	26.5	36	22.1	24	23.1	
Unknown	42	10	7.6	16	9.8	16	10.2	
ECOG 0	106	42	31.8	30	18.4	34	29.3	.009[c]
1	75	21	15.9	36	22.1	18	15.5	
2	48	15	11.4	28	17.2	5	4.3	
3	17	6	4.6	6	3.7	5	4.3	
4	2	1	0.8	0	0.0	1	0.9	
Unknown	163	47	35.6	63	38.6	53	45.7	

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TABLE 3
Patient and tumor characteristics (n=411) (continued)

	Total (n=411) n	p16+/p53− (n=132) n	%	p53+ (n=163) n	%	p16−/p53− (n=116) n	%	P value
Type of vulvar surgery								.638[c]
Wide excision	39	11	8.3	14	8.6	14	12.1	
Partial vulvectomy	130	47	35.6	52	31.9	31	26.7	
Complete vulvectomy	194	59	44.7	78	47.6	57	49.1	
Exenteration	3	2	1.5	0	0.0	1	0.9	
Surgery type unknown	35	9	6.8	14	8.6	13	10.3	
No surgery	10	4	3.0	5	3.1	1	0.9	
Resection status R0	281	95	72.0	108	66.2	78	67.2	.100[c]
R1	52	18	13.6	24	14.7	10	8.6	
Rx (including no surgery, n=10)	68	19	14.4	31	19.0	28	24.1	
Type of groin surgery								.397[c]
LAE performed	304	99	75.0	115	70.5	90	77.6	
SLN procedure	81	19	14.4	40	24.5	22	18.9	
No LAE/unknown	107	33	25.0	48	29.4	26	22.4	
radiotherapy during primary treatment								.496[c]
Yes	126	38	28.8	57	34.9	32	27.5	
Including vulva	98	34	25.7	40	24.5	25	21.5	
No	250	86	65.2	89	54.6	72	62.1	
Unknown	35	8	6.1	17	10.4	12	10.3	

ANOVA, analysis of variance; *ECOG*, Eastern Cooperative Oncology Group; *HPV*, human pappilomavirus; *hr*, high risk; *SD*, standard deviation; *SLN*, sentinel lymph-node; *UICC*, Union for International Cancer Control.

[b] 6th edition of UICC TNM staging system; *P* values from: [a] ANOVA; [c] Pearson chi-square; [d] Fisher's exact test; [e] Owing to poor DNA quality.

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TABLE 4
Site of disease recurrence with regard to expression of p16 und p53

Localization of disease recurrence	Total (n=411)		p16+p53− (n=132)		p53+ (n=163)		p16−/p53− (n=116)	
Recurrent disease	122	29.7%	30	22.7%	57	34.9%	35	30.2%
Vulva (± other localizations)	86	17.8%	19	14.4%	31	19.1%	22	18.9%
Vulva only	64	15.6%	16	12.1%	27	16.6%	19	16.4%
Groins only	13	3.2%	1	0.8%	8	4.9%	4	3.5%
Pelvis/distant (± other localizations)	34	8.3%	10	7.6%	17	10.4%	8	6.9%
Unknown	2	0.5%	0	0.0%	1	0.6%	1	0.9%
Death before recurrence	57	13.9%	11	8.3%	26	15.9%	20	17.2%

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that presented *TP53* wild-type gene and HPV negativity was found. For the 3 *TP53* wild-type/HPV-negative samples, mutations in *SYNE1, NOTCH1, KMT2C, FGFR3, KMT2D, FBXW7,* and *POLE* were detected.[8] Potential explanations for a double negative expression pattern include aging of the tissue. However, the proportion of negative cases did not decrease significantly over time looking at our whole cohort. More importantly, a double negative expression pattern can be caused by *TP53* nonsense mutations or deletions which can result in a p53 null phenotype, showing no p53 staining in the tumor cells with a consecutively p53 null phenotype showing no p53 staining in any tumor cell. The proportion of tumors with immunohistochemically "hidden" *TP53* mutation in the p53−/ p16− subgroup can only be estimated at this point; the rates reported in the currently available sparse literature vary between 50% and 80%.[9,32] Consequently, we excluded those cases from our analysis. However, nearly 30% of the tumors in our analysis still showed a p53− (wild-type)/p16− pattern with an intermediate prognosis. A just recently published analysis by Kortekaas and colleagues[33] indicated similar results, although they included p53 completely negative cases in the p53 abnormal group: Although HPV+ VSCC showed the best clinical outcome, HPV−/p53 wild-type cases showed an intermediate prognosis in comparison with p53 abnormal cases.

Generally, somatic mutations occur more often in HPV-negative than HPV-positive vulvar cancer.[22] *TP53* gene mutations are the most frequently described mutations in HPV-independent disease, "compensating" for the absence of HPV E6. Mutations can already be found in precursor lesions and lichen sclerosus, indicating that p53 alterations are involved early in carcinogenesis of HPV-independent disease. An analysis by Trietsch et al[7,36,37] summarizes 34 articles investigating somatic mutations in VSCC: The observed incidence of *TP53* mutations was up to 81%. *TP53* mutations cause a high instability of proteins leading to an overexpression of p53 and consequently to a dysregulation of cell cycle with uncontrolled cellular proliferation. Reports on the correlation between p53 expression and prognosis in VSCC have been contradictory[38]: In a retrospective single-center analysis of 97 vulvar carcinomas, 3 independent predictors for an improved OS were identified: absence of p53 expression (P=.02), early tumor stage (P<.006), and p16 expression (P=.002).[27] The 5-year survival rate for IHC p53 positive cancers was 36% compared with 68% of p53 negative cancers. However, other studies failed to show a correlation between the expression of p53 and PFS or OS in patients with VSCC.[37,39,40] Our large analysis now confirms a negative prognostic role of p53 overexpression with p53+ tumors being more often node positive. With regard to HPV detection, the rate of HPV DNA positive tumors

was high but in the range of previously reported positivity rates,[22] especially for a comparably young cohort like ours (median age, 67.8 years) and with the p53 silent tumors excluded from analysis. Interestingly, 4.6% of the tumors with positive p16 IHC were HPV DNA negative after repeated testing. These tumors are most likely HPV independent and p16 overexpression attributable to a CDKN2A or RB1 mutation as previously described in head and neck squamous cell carcinoma (HNSCC), but HPV-positive cases with deletions resulting in false negative PCR cannot be excluded.[41] With regard to prognosis, we could not demonstrate a difference between p16+/HPV+ and p16+/HPV− cases, with the limitation of the small numbers in the latter group. Similarly, we could not demonstrate a prognostic role of HPV PCR results in the p16− subgroups. The observed detection rates of HPV DNA in the p16− subgroups (32%) are comparable with those previously observed by other groups in smaller cohorts of HNSCC.[42] Consequently, it has to be proposed that HPV PCR alone is not suitable to discriminate prognostic groups in VSCC and must either be combined with p16 IHC or p16 IHC could even be sufficient alone.

Clinical implications
In VSCC, more than two-thirds of all cases can be cured by surgery alone; therefore, deescalation of treatment in analogy to HPV-driven HNSCC is a difficult task. Escalation of treatment in

p53 positive cases is possible but often hampered by age and performance of this subgroup of patients. In a first step, p16/p53 IHC as a widely recognized marker should be performed in each VSCC specimen. Even though p53/p16 IHC might not yet guide treatment decisions, follow-up for example could be more stringent in p53 positive disease.

Research implications

The clinically relevant third subgroup identified in our study with a p53−/p16− phenotype showing an intermediate prognosis needs to be further characterized in the future. A next consecutive step seems to be the analyses of the *TP53* mutational status of the p53−/p16− cohort to report on the quantity of biologically relevant *TP53* knockout mutations and their clinical relevance. Furthermore, molecular panel analyses will help to further characterize this subgroup of patients.

Strengths and limitations

A major strength of the presented data is the large multicentric character of the study with a clinically well characterized patient cohort.

A relevant limitation of our study is the short duration of median follow-up. However, it is unlikely that the prognostic differences between the groups will be graded with longer duration of follow-up because recurrences critical for survival (especially nodal recurrences) occur early in the course of the disease.[43] The study is further limited by the questions of discrepancies between p16 IHC positivity and HPV detection via PCR and lack of p53 IHC detection owing to potential knockout mutations that remains unclear at this point.

Conclusion

P53 and p16 expression profiles reveal 3 prognostic relevant subtypes in vulvar cancer. Although p16 overexpression is associated with an improved prognosis, p53 overexpression is linked to an adverse outcome in VSCC. Our data provide further evidence of a clinically

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FIGURE 2
Survival with regard to expression profile
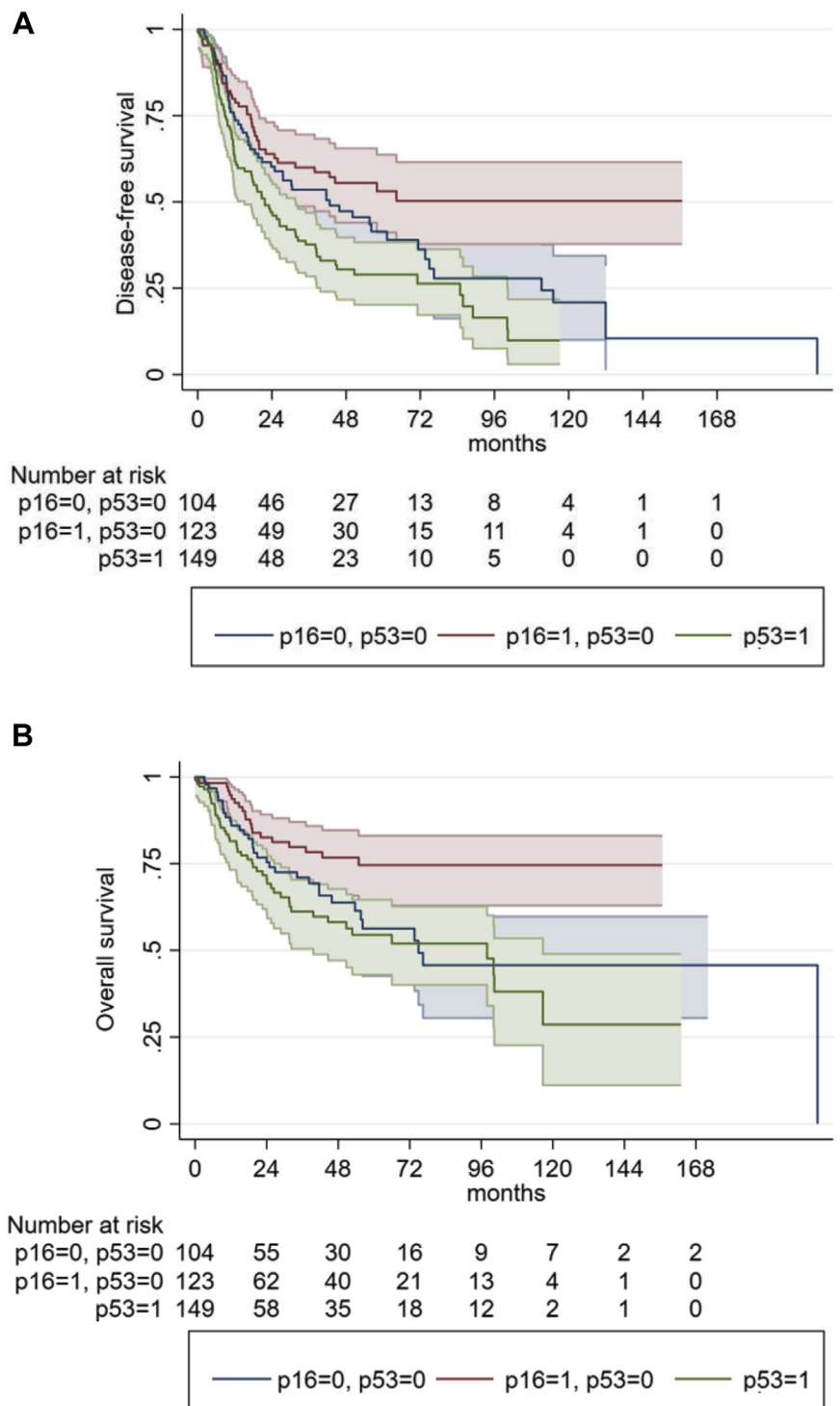
A, DFS with regard to expression profile. **B,** OS with regard to expression profile.

DFS, disease-free survival; *OS,* overall survival.

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TABLE 5
Multivariate analyses of variables influencing DFS (n = 376; number of events = 177)

	HR	*P* value	95% CI	
p16+p53− vs p53+	0.66	.042	0.439	0.986
p16−/p53− vs p53+	0.78	.197	0.540	1.136
Age (per y)	1.03	<.001	1.016	1.046
pT2 vs pT1b	1.41	.076	0.964	2.073
pT3/pT4 vs pT1b	1.65	.063	0.973	2.800
pT unknown vs pT1b	1.716	.332	0.576	5.112
pN1 vs pN0	2.22	<.001	1.600	3.095
Grade 2 vs grade 1	0.83	.441	0.507	1.344
Grade 3 vs grade 1	0.93	.789	0.527	1.627
Grade unknown vs grade 1	0.39	.286	0.071	2.186
R0 vs R1	0.60	.020	0.390	0.922
Rx vs R1	0.50	.024	0.279	0.912
ECOG 1 vs 0	2.14	.004	1.276	3.599
ECOG 2 vs 0	1.58	.113	0.897	2.789
ECOG 3/4 vs 0	2.43	<.001	1.549	3.821

CI, confidence interval; *ECOG*, Eastern Cooperative Oncology Group; *HR*, hazard ratio.

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relevant third subgroup of VSCC with a p53− (wild-type)/p16− phenotype showing an intermediate prognosis that needs to be further characterized. ∎

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