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CDKN2A(p16) and *HRAS* are frequently mutated in vulvar squamous cell carcinoma

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HIGHLIGHTS

- 107 vulvar squamous cell carcinomas were screened for somatic mutations and HPV infection.
- 62% of the tumours carried at least one mutation, with TP53, CDKN2A and HRAS being the most frequent.
- Patients with a somatic mutation, and specifically *HRAS* mutations, have a worse prognosis.

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ABSTRACT

Background. Two etiologic pathways of vulvar cancer are known, a human papillomavirus (HPV)- and a *TP53*-associated route, respectively, but other genetic changes may also play a role. Studies on somatic mutations in vulvar cancer other than *TP53* are limited in number and size. In this study, we investigated the prevalence of genetic mutations in 107 vulvar squamous cell carcinomas (VSCCs).

Methods. A total of 107 paraffin-embedded tissue samples of primarily surgically treated VSCCs were tested for HPV infection and screened for mutations in 14 genes (*BRAF*, *CDKN2A*(p16), *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, *PTEN*, and *TP53*) using Sanger sequencing and mass spectrometry.

Results. Mutations were detected in 7 genes. Of 107 VSCCs, 66 tumors (62%) contained at least one mutation (*TP53* = 58, *CDKN2A*(p16) = 14, *HRAS* = 10, *PIK3CA* = 7, *PPP2R1A* = 3, *KRAS* = 1, *PTEN* = 1). Mutations occurred most frequently in HPV-negative samples. Five-year survival was significantly worse for patients with a mutation (47% vs 59%, $P = .035$), with a large effect from patients carrying *HRAS*-mutations.

Conclusion. Somatic mutations were detected in 62% of VSCCs. As expected, HPV infection and *TP53*-mutations play a key role in the development of VSCC, but *CDKN2A*(p16), *HRAS*, and *PIK3CA*-mutations were also frequently seen in HPV-negative patients. Patients with somatic mutations, especially *HRAS*-mutations, have a significantly worse prognosis than patients lacking these changes, which could be of importance for the development of targeted therapy.

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Introduction

Vulvar cancer is the fourth most common gynecological malignancy with an incidence of 2 per 100,000 women each year in developed countries [1]. This cancer is usually seen in post-menopausal women; the average age at diagnosis is 70 years [2]. Although most patients can be treated curatively by radical local excision, this surgical therapy can be very mutilating and results in high morbidity rates [3–5]. Almost 90% of all vulvar carcinomas are squamous cell carcinomas (SCCs) [6], which can be divided into two different etiological types [7]. The first type occurs mainly in younger patients and is clearly associated with high-risk human papillomavirus (hrHPV) infection and the precursor lesion usual vulvar intraepithelial neoplasia (uVIN) [8]. The second type is typically seen in elderly patients and seems to develop independently from HPV infection. The HPV-independent type of carcinoma is associated with lichen sclerosis and mutations in the *TP53* gene, but its etiology is much less well understood than the HPV-positive type [7,8].

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Studies on somatic mutations in vulvar cancer other than in the *TP53* gene are limited in number and size. As shown in studies concerning other types of cancer, information about the role of genetic mutations may help us understand the processes underlying vulvar cancer. In addition, mutation profiling may guide targeted therapies in cancer [9–12]. If mutational status has prognostic significance, we might be able to select those patients who need a more radical approach versus those who can be spared the morbidity of extensive radical surgery or additional radiotherapy and/or chemotherapy. Somatic mutations have been studied extensively in other types of gynecological cancer and in squamous cell carcinoma (SCC) of the skin [13]. Based on this knowledge, we have selected the genes that are most frequently affected in gynecological cancer and SCC of the skin that could play a role in the tumorigenesis of vulvar cancer [14]. In this study, we investigated the prevalence of somatic mutations in 14 different genes in 107 vulvar squamous cell carcinomas (VSCCs) and correlated these changes with survival.

Methods

Patients

Clinical and follow-up data were retrieved from patient medical records and the cancer registration database for patients who were surgically treated for primary VSCC between 2000 and 2009 in the Leiden University Medical Center, Leiden, the Netherlands, a referral center for gynecological cancers. Patients were excluded if they had received chemotherapy or radiotherapy in the pelvic area before surgery. Tumors from which only biopsies were taken were excluded from the series. Tumor staging was performed according to the FIGO system; we used the 1995 staging instead of the revised 2009 staging because of the retrospective design of the study [15,16]. Follow-up ended in December 2012. Patient samples were handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies. (www.federa.org, an English translation of the Code can be found here: http://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf). Patients received information on the secondary use of tissue that is sampled for diagnostic use. They can actively object to secondary use.

Therapy

Patients were generally treated according to Dutch national and international vulvar cancer guidelines [3]. Patients with unifocal VSCC with a diameter <4 cm without suspicious groin nodes were generally treated with radical local excision (RLE) and a sentinel node procedure. Sentinel node mapping was performed using Technetium-99 m nanocolloid combined with patent blue [17]. Tumors with a diameter exceeding 4 cm or multifocal tumors were generally treated with RLE and unilateral or bilateral inguinofemoral lymphadenectomy. In patients with tumors in FIGO stage III or higher but with contraindications for extensive surgery, such as high age and comorbidity, RLE was performed without inguinofemoral lymphadenectomy, followed by (chemo)radioation.

Immunohistochemistry

Sections of 4 μm thick were taken from formalin-fixed, paraffin-embedded (FFPE) tissue blocks from selected patients with primary VSCC. Tissue sections were stained as described previously [18] using primary antibodies for pankeratin AE1/AE3 (clone MAB3412, 1:2000, Millipore, Billerica, Massachusetts, USA) for the selection of tumor area and for PTEN (clone 6H2.1, 1:80, Dako, Glostrup, Denmark) to additionally assess aberrant expression of the protein.

PTEN protein expression scoring was performed blinded for clinical data and for the results from the mutation analysis. PTEN expression was scored as positive or negative, with adjacent normal epithelium and stroma as an internal positive control. Tumors with areas of markedly decreased or absent PTEN expression were considered to have aberrant expression.

DNA isolation

The pankeratin-stained slides were used to select an area consisting of at least 70% tumor cells. Three 0.6-mm diameter tissue cores of variable length were taken from the selected area in the FFPE blocks. DNA isolation was performed in an automated fashion as described previously using the Tissue Preparation System (Siemens Healthcare Diagnostics, Malvern, Pennsylvania, USA) [19]. DNA quality was tested by multiplex quality PCR that amplified 150-, 255-, 343-, and 511-base pair products that were visualized using 2% agarose gel electrophoresis and scored for quality (scale, 0–4) (primer sequences available upon request).

HPV analysis

DNA extracted from two 10-μm whole tissue sections was used for HPV type analysis. Sections of a paraffin block without tissue were cut before and after each tumor sample to prevent contamination and served as a negative control. All blank paraffin sections were negative in the PCR analysis. The INNO-LiPA HPV Genotyping *Extra* Amp kit for in vitro diagnostic use (Innogenetics, Gent, Belgium), a highly sensitive hybridization assay, was used for HPV typing as described previously [20]. This assay can detect oncogenic and common HPV types [20].

Mutation genotyping using MALDI-TOF mass spectrometry

Mutation genotyping was performed using the GynCarta 2.0 panel [14], which contains several loci on 13 genes (*BRAF*, *CDKN2A*(p16), *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A* and *PTEN*), following the manufacturer's protocol as described previously [21]. Briefly, wild-type and mutant DNA was amplified by multiplex PCR. Shrimp alkaline phosphatase treatment inactivated surplus nucleotides. A primer extension reaction (iPLEX® Pro) was performed with mass-modified terminator nucleotides, and the product was spotted on a SpectroCHIP (Sequenom, Hamburg, Germany). The distinct masses were determined with MALDI-TOF mass spectrometry.

All 107 tumor samples were genotyped and 11 (10%) samples were assayed in duplicate. Non-template ($N = 4$) and wild-type leukocyte DNA ($N = 2$) controls were included in each multiplex to obtain negative and wild-type MALDI-TOF spectra.

Analyzing TP53 mutations

For analysis of somatic mutations in the *TP53* gene, DNA sequencing was performed for exons 5–8. For each sample, 2 μL (5 ng/μL) DNA was added to a 23-μL PCR mix containing 12.5 μL SYBRgreen (Bio-Rad, Hercules, Callifornia, USA), 0.6 μL (10 pg/μL) of each primer (sequences are available upon request), and 9.9 μL demineralized water. Real-time PCR was performed for 40 cycles using an annealing temperature of 60 °C. For each exon, one wild-type leukocyte was used as a quality control. All PCR products were purified by a vacuum using MinElute 96-well plates from the PCR purification kit (Qiagen, Hilden, Germany). Sequencing was performed for both forward and reverse strands by Macrogen Europe (Amsterdam, the Netherlands).

Data analysis

Data were analyzed with MassARRAY Typer Analyser software (TYPER 4.0.22, Sequenom, Hamburg, Germany) and MutationSurveyor

(Softgenetics, State College, Pennsylvania, USA). Three investigators blinded to tumor identification inspected the cluster plots and mutation calls to discriminate true mutant calls from artifacts, and a consensus determination was reached.

Statistical analysis

Statistical analyses were conducted using the Predictive Analytics Software package (version 17, IBM-SPSS Statistics, Armonk, New York, USA). The independent *t*-test was used to compare baseline variables and Fisher's exact test to analyze categorical and normally distributed numerical data. We chose to also compare the separate characteristics that make up the FIGO staging, because these characteristics are unaffected by differences between the old and new FIGO staging systems. The Shapiro–Wilk test was used to test for normality, and for data with a skewed distribution, the Mann–Whitney *U* test was used. Kaplan–Meier, the log-rank test, and Cox proportional hazard regression analysis including age, HPV infection, tumor size, and the most frequent mutations in this cohort were performed to analyze differences in survival between groups of patients with and without genetic mutations. A *P* value of .05 was considered significant, corresponding to 95% confidence intervals (CIs). All tests were two-tailed. Results for normally distributed numerical data are presented as the mean with standard deviation (SD), and results for skewed numerical data are presented as the median with interquartile range.

Disease-free survival was defined as survival from the date of surgery until the first recurrence or death or until the end of study follow-up. The overall survival of the patients was measured from surgery until death from any cause or end of study follow-up, and disease-specific survival was measured from the date of surgery until death from vulvar cancer or the end of study follow-up. Recurrent disease in the vulvar area was characterized as ‘local recurrence’ whereas a recurrence in the inguinal region was characterized as ‘regional recurrence’. Recurrent disease on the contralateral side of the vulva was considered to be a second primary tumor. Remaining recurrences were documented as distant recurrences.

Table 1
Characteristics of the patients with vulvar squamous cell carcinoma in this study.

Characteristic			Value	
Follow up[a]	– mo	(IQR)	38.0	(16.0–69.0)
Age at diagnosis[b]	– year	(SD)	70.1	(14.0)
Duration of symptoms[a]	– mo	(SD)	5.0	(2.0–18.5)
FIGO stage	– n	(%)		
Stage 1			29	(27.1)
Stage 2			36	(33.6)
Stage 3			30	(28.0)
Stage 4			12	(12.0)
Treatment	– n	(%)		
Radical vulvectomy			68	(63.6)
Radical local excision			39	(36.4)
Adjuvant radiotherapy			43	(40.2)
Adjuvant chemotherapy			1	(0.9)
HPV positive	– n	(%)	18	(16.8)
Lymph node metastases	– n	(%)	41	(38.3)
Unilateral			28	(26.2)
Bilateral			13	(12.1)
Extracapsular growth			17	(15.9)
Tumor size[b]	– mm	(SD)	32.1	(22.2)
Infiltration depth[a]	– mm	(IQR)	6.0	(4.0–11.0)
Vasoinvasion	– n	(%)	15	(14.0)
Lymfangioinvasion	– n	(%)	3	(2.8)
Perineural growth	– n	(%)	4	(3.7)
Positive resection margins	– n	(%)	21	(19.6)
Disease status	– n	(%)		
Patients in complete remission			82	(76.6)
Local recurrence			22	(20.6)
Second primary tumour			9	(8.4)
Regional recurrence			9	(8.3)
Died			59	(55.1)
Disease specific death			28	(26.2)
Regional metastases			8	(7.5)
Distant metastases			24	(22.4)
5-year overall survival	– %	(SD)	50.1	(5.1)
5-year disease specific survival	– %	(SD)	67.8	(6.3)
5-year disease free survival	– %	(SD)	27.9	(5.0)

Abbreviations: N = number; mo = months.
[a] Median (interquartile range, IQR).
[b] Mean (standard deviation, SD).

Results

Between January 2000 and December 2009, 129 patients were surgically treated for primary VSCC at the Leiden University Medical Center. Nine patients were excluded because they had a history of chemotherapy, pelvic radiotherapy, or immunosuppressive therapy for vulvar cancer or any other disease prior to the current diagnosis. Eleven patients were excluded because only a biopsy of their tumor was taken and no further surgical treatment was given. Tumor tissue from one patient was of poor quality and was not analyzed further. Thus, a total of 108 patients with surgically treated primary VSCC met all of our inclusion criteria. This cohort of patients has been described before [22].

Of 108 samples, one was of such low DNA quality that it failed in all tests, and we excluded this patient from further analyses. Table 1 lists the characteristics for the included patients.

Of 107 samples, 18 (17%) were positive for hrHPV and 66 (62%) carried at least one mutation. Of these patients, 16 (15%) had 2, and 7 (6.5%) had 3 different mutations. Fifty-eight (54%) patients harbored a mutation on *TP53*. Sequencing for *TP53* repeatedly failed for five patients. Fourteen patients carried a *CDKN2A*(p16) mutation, 10 an *HRAS* mutation, *PIK3CA* was found in 7 patients, mutated *PPP2R1A* was identified in three samples, and *KRAS* and *PTEN* were each mutated in one patient. The most frequent combination of mutations was *TP53* and *CDKN2A* (11 patients, 10.3%), followed by *TP53* and *HRAS* (7 patients, 6.5%). Additional immunohistochemistry revealed aberrant PTEN expression in another five samples. Fig. 1 provides a graphic representation of the mutation spectrum. Supplemental Table 1 lists the amino acid and base pair changes for each patient. Of the 18 patients that tested positive for high-risk HPV, three also had a somatic mutation (all *TP53*, Table 2).

Correlating clinical data with mutation status

A comparison between patients with a mutation in one of the tested genes compared to those without mutations was made (Table 3). Patients with at least one mutation in their tumor sample were significantly older (73 vs 65 years, $P = .002$) and had larger tumors (36 mm vs 26 mm, $P = .024$) with deeper infiltration of the tumor (7.5 mm vs 5.0 mm, $P = .003$). Tumor size was correlated with depth of infiltration (Pearson Correlation .595, $P < .001$). Not having an HPV infection was clearly associated with the detection of somatic mutations: Of the 18 HPV-infected patients, only 3 (4.5%) had a somatic mutation (all *TP53*) whereas in non-HPV-infected patients, 63 out of 89 carried a mutation (70.8%, $P < .001$)

Survival analysis

Patients carrying at least one mutation had a significantly worse overall 5-year survival (46.9% vs 58.9%, log-rank $P = .035$). Disease-specific 5-year survival was 67.8% in patients carrying a mutation versus 80.4% in patients without mutations (log rank $P = .058$) (Fig. 2). Disease-free survival was comparable for patients with and without mutations (37.8% vs 43.6%, log rank $P = .472$). Cox regression analysis for disease specific survival including age, HPV infection, tumor size, and the three most frequent mutations in this cohort (*TP53*, *CDKN2A*,

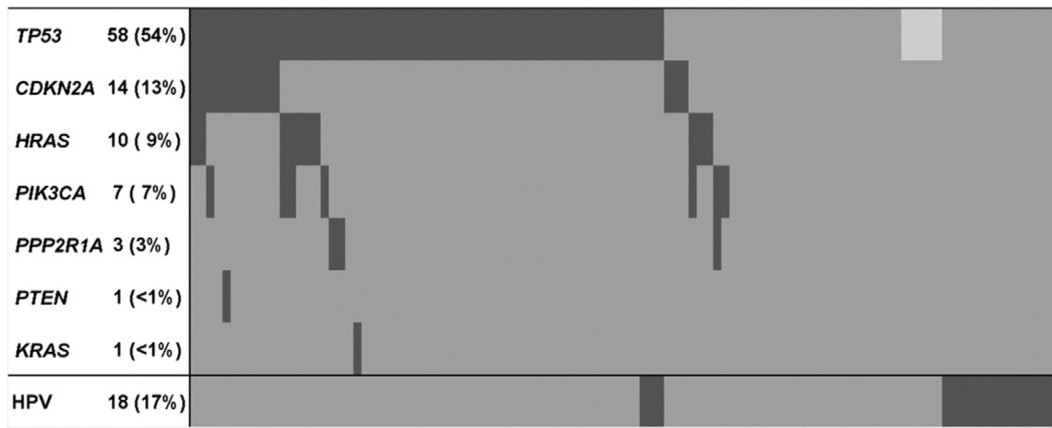

Fig. 1. Mutation spectrum. Mutation spectrum for 107 VSCC patients. Medium grey = wild type or non-infected. Dark grey = mutated or infected. Lightest grey = assay failed.

and *HRAS*), showed a significantly worse prognosis for patients carrying an *HRAS* mutation (hazard ratio 2.9, 95% CI 1.112–7.425). HPV infection and *CDKN2A* or *TP53* mutations were not independent prognostic factors (Table 4). Interestingly, although the absolute number of coexisting mutations did not correlate with an increased hazard ratio for disease-specific death, having both an *HRAS* and a *TP53* mutation or both a *CDKN2A* and a *TP53* mutation increased the hazard ratios to 5.1 (95% CI 1.504–17.062) and 4.1 (95% CI 1.2–14.074), respectively, when correcting for HPV infection, tumor size, and age.

Discussion

The present results confirm the hypothesis that HPV infection and *TP53* mutations play a key role in the development of VSCCs. In agreement with current understanding of the pathogenesis of vulvar cancer [23], the two pathways (HPV and non-HPV related) could clearly be distinguished in this cohort. While patients infected with high-risk HPV were significantly less likely to carry somatic mutations, mutations were not exclusively found in HPV-negative patients. Our results show that not only *TP53* plays a major role in HPV-negative VSCC patients but that *HRAS*, *CDKN2A*(p16), and to a lesser extent *PIK3CA* are also frequently mutated. A mutation in VSCC in *CDKN2A* has been described previously [24], but the finding of mutations in *HRAS*, *PIK3CA*, *CTNNB1*, *PPP2R1A1*, and *KRAS* has, to our knowledge, not been reported before in vulvar cancer.

Following the currently accepted two-pathway etiology of VSCC, vulvar carcinogenesis is either driven by a persistent HPV infection or by a somatic mutation, most often in the *TP53* gene. In contrast to this hypothesis, in our analysis, we found a subgroup of patients (n = 26, 25%) with neither any somatic mutation in our gene panel, nor an HPV infection. As any other technique, sequencing and mass spectrometry have their weaknesses. An asset of the mass spectrometry technique we have used is that it works well with low quality DNA, and that it requires mutant allele proportions as low as 5%. Our tumor samples contained at least 70% tumor cells, so we have confidence that the mutations in our panel can be detected if present. We are aware of the fact that these detection techniques have an acceptable, but no complete coverage of the genes we have studied. We therefore expect that the actual number of somatic mutations in VSCC might be slightly higher than we have detected, and that a part of the 26 patients without mutations or HPV, actually do carry a somatic mutation that we cannot pick up using our panel. Sequencing the full length of all the genes we have selected in this study would be very interesting, but would also require large amounts of high quality DNA. Unfortunately however, FFPE material is often of limited quantity and quality and therefore full sequencing is not possible.

One study [25] found that five out of eight patients (63%) in their study harbored *PTEN* mutations. Using hot-spot mutation analysis covering the same mutations that Holway et al. found, we detected no more than one mutation in *PTEN*. This divergence could partially be explained by the fact that the panel we used can detect only approximately 40% of all known *PTEN* mutations. We also applied immunohistochemistry to complement the *PTEN* data but found no more than 5% of the tumors showing loss of PTEN expression. Because PTEN is often downregulated by epigenetic mechanisms rather than by somatic mutations alone, the aberrant PTEN expression is likely not attributable to somatic mutations in all five samples [26]. Our data thus suggest that the results by Holway et al. might have given an overestimation of the prevalence of *PTEN* mutations in VSCCs.

Our results could help clinicians in differentiating high-risk patients with a worse prognosis who would benefit from closer follow-up and radical surgery or adjuvant treatment, from low-risk patients that can be treated less aggressively with resulting less morbidity. Furthermore, clinical trials have shown promising outcomes of targeted therapies, such as in targeting the *PI3K*/AKT/mTOR pathway in colorectal cancer [10]. The finding that *HRAS*, and to a lesser extent *PIK3CA*, are frequently mutated in vulvar cancer could be of great importance in the development of new treatment strategies, and individualized treatment. Our study revealed RAS mutations in 11 out of 107 patients (10 *HRAS*, 1 *KRAS*). Seven of our patients were found to have a mutation in the oncogene *PIK3CA*, which is a downstream effector of the RAS pathway. Monotherapy of PI3K inhibitors has demonstrated poor clinical efficacy, likely due to adaptive resistance of the tumor cells [27]. It seems that a dual blockade of upstream RAS by MEK inhibitors, and a more downstream inhibition of PI3K, may have better clinical results than single pathway blocking treatment [28]. Although a simultaneous mutation

Table 2
Somatic mutations detected in hrHPV positive and hrHPV negative patients.

Gene	hrHPV negative		hrHPV positive	
	n = 89	(83%)	n = 18	(17%)
BRAF	0	(0)	0	(0)
CDKN2A	14	(16)	0	(0)
CTNNB1	0	(0)	0	(0)
HRAS	10	(11)	0	(0)
KRAS	1	(1)	0	(0)
NRAS	0	(0)	0	(0)
PIK3CA	7	(8)	0	(0)
PTEN	1	(1)	0	(0)
PPP2R1A	3	(3)	0	(0)
TP53	55	(62)	3	(17)

Abbreviations: hrHPV = high risk Human Papilloma Virus.

Table 3
Comparison of the clinical outcomes for patients with and without somatic mutations.

Outcome			1 ≥ mutation		No mutation		*P* value
			n = 66	(62%)	n = 41	(38%)	
Follow-up[a]	– mo		34.5	(11.0–64.3)	55.0	(25.0–80.0)	0.043[b]
Age at diagnosis[c]	– year		73.4	(11.9)	64.9	(15.6)	0.002[b]
Duration of symptoms[a]	– mo		4.0	(2.0–18.5)	6.0	(2.5–19.0)	0.068
FIGO stage	– n	(%)					0.051
Stage 1			12	(18.2)	17	(41.5)	
Stage 2			23	(34.8)	13	(31.7)	
Stage 3			22	(33.3)	8	(19.5)	
Stage 4			9	(13.6)	3	(7.3)	
Treatment	– n	(%)					
Radical vulvectomy			45	(68.1)	23	(56.1)	0.245
Radical local excision			21	(31.8)	18	(43.9)	
Adjuvant radiotherapy			30	(45.5)	13	(31.7)	0.222
Adjuvant chemotherapy			1	(1.5)		(0.0)	
HPV positive	– n	(%)	3	(4.5)	15	(36.6)	<0.001[b]
Lymph node metastases	– n	(%)	30	(45.5)	11	(26.8)	0.067
Unilateral			20	(30.3)	8	(19.5)	0.148
Bilateral			10	(15.2)	3	(7.3)	
Extracapsular growth			14	(21.2)	3	(7.3)	0.063
Tumor size[c]	– mm		36.0	(22.8)	26.1	(20.1)	0.024[b]
Infiltration depth[a]	– mm		7.5	(5.0–11.0)	5.0	(2.0–7.0)	0.003[b]
Vasoinvasion	– n	(%)	10	(15.2)	5	(12.2)	0.779
Lymfangioinvasion	– n	(%)	2	(3.0)	1	(2.4)	1.000
Perineural growth	– n	(%)	3	(4.5)	1	(2.4)	1.000
Positive resection margins	– n	(%)	15	(22.7)	6	(14.6)	0.453
Disease status	– n	(%)					
Complete remission			46	(69.7)	36	(87.8)	0.036[b]
Local recurrence			14	(21.2)	8	(19.5)	1.000
Second primary tumour			7	(10.6)	2	(4.9)	0.544
Regional recurrence			2	(3.0)	2	(4.9)	0.877
Died			41	(62.1)	18	(43.9)	0.075
Disease specific death			21	(37.5)	7	(20.6)	0.106
Regional metastases			9	(13.6)	6	(14.6)	1.000
Distant metastases			16	(24.2)	8	(19.5)	0.639
5-year overall survival	– %	(SD)	46.9	(6.4)	58.9	(8.0)	0.035[b]
5-year disease specific survival	– %	(SD)	67.8	(6.0)	80.4	(6.7)	0.058
5-year disease free survival	– %	(SD)	37.8	(8.1)	43.6	(8.8)	0.472

Abbreviations: N = number; mo = months.
[a] Median (interquartile range, IQR).
[b] Significant difference ($P < .05$).
[c] Mean (standard deviation, SD).

in both *HRAS* or *KRAS* and *PIK3CA* occurred in only 3% of our patients, both these patients and those with a single mutation in either of these genes may benefit from this dual targeted therapy. Research in other tumor types has shown that also in *PIK3CA* wild type, *KRAS* mutated colorectal cancers, a combination of PI3K/MEK inhibitors results in greater tumor regression than treatment with one inhibitor alone [29]. These targeted therapies may be of special interest for the subgroup of patients that currently cannot be treated by surgery alone or by surgery at all, such as patients with inoperable distant metastasis and tumors with spread into for example the rectum or bladder, or patients that are unfit for surgery because of comorbidities or their high age.

Fourteen patients (13%) in our cohort tested positive for mutations in *CDKN2A*. *CDKN2A*, encoding the proteins p16^{INK4A} and p14^{ARF}, is a tumor suppressor gene that regulates the cell cycle by decelerating cells progression from G1 phase to S phase [30]. It functions as a stabilizer of p53. *TP53* is mutated in many different types of cancer and

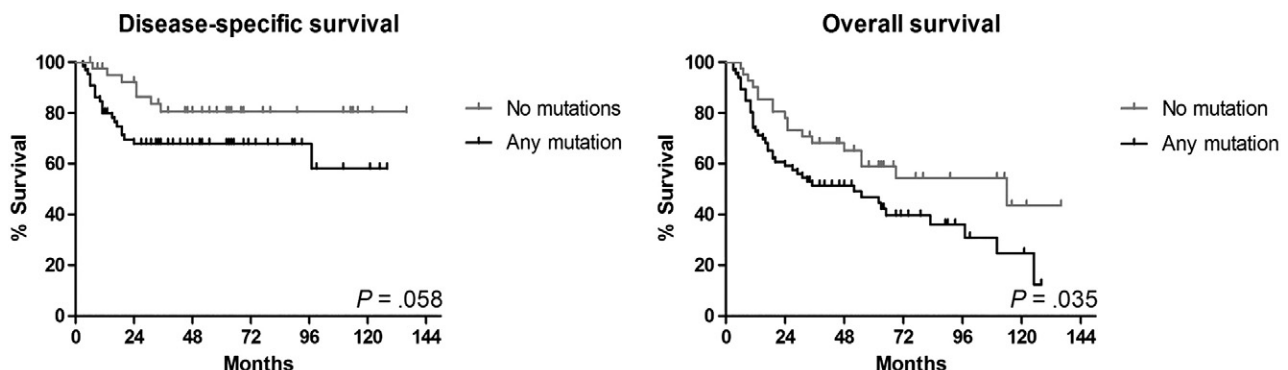

Fig. 2. Survival curves. Overall survival, $P = .035$ (left), and disease-specific survival, $P = .058$ (right) for 107 vulvar squamous cell carcinoma patients in this study. Upper line (grey) = no mutation detected; lower line (black) = mutation detected.

Table 4
Cox regression analyses for disease specific survival.

Variable	Hazard ratio	95% CI	*P* value
Age (years)	0.980	0.948–1.013	.232
HPV infection	<0.001	NA	.963
Tumor size (mm)	1.022	1.006–1.039	.007
TP53	1.743	0.728–4.173	.212
CDKN2A	2.301	0.830–6.376	.109
HRAS	2.848	1.113–7.290	.029

CI = confidence interval.

assumed to be a driver mutation in the majority of these tumors. In non-cancerous cells, *TP53* stops cells from dividing when aberrations in the DNA are detected. Loss of normal p53 function leads to an uncontrolled cell cycle and chromosomal instability [31]. Research in other types of cancer such as head and neck SCC and breast cancer have reported a correlation between *TP53* mutations and a worse survival [32,33]. In contrast, *TP53* was not an independent prognostic factor in the multivariate survival analysis in our cohort. We found that patients with *TP53* mutations frequently carried additional mutations and that the combination of a mutation in both *TP53* and *HRAS* or *CDKN2A* correlated with a significantly worse prognosis. When both *CDKN2A* and *TP53* are mutated, the cascade of destabilization of the tumor cells progresses, and might indeed explain the worse survival of patients carrying both mutations. The worse survival in patients carrying *TP53* and *HRAS* is in line with those of Zhang et al. who showed a markedly increased number of tumors and tumor size in transgenic mice with *TP53* and *HRAS* mutations [34]. The synergistic interaction and the resulting impairment of prognosis could be explained by the roles that both genes have in tumorigenesis. *HRAS* is a proto-oncogene, that, when mutated, directs cell growth and division [28]. Since *TP53* is a tumor suppressor gene regulating cell cycle control, the combination of these mutations set the stage for uncontrolled cell division. Another explanation could be that *TP53* mutated tumors are simply more susceptible to additional mutations because of a lack of cell cycle control. The reported worse prognosis in tumors with multiple mutations could therefore also be a reflection of further progression of the tumor.

The fact that additional mutations in *CDKN2A* and *HRAS* occur frequently makes us question why there is a specific preference for these two accessory mutations and whether *TP53* mutations truly are the drivers of aggressive tumor growth in VSCCs, or that this aggressiveness is caused by the additional mutations that occur later on in tumor progression. A survival analysis stratified for different types of p53 protein activity changes and gene location, and for the low frequent somatic mutations that we found would be very interesting but requires study cohorts much larger than the 107 we studied here. The same thing applies to multivariate survival analyses: because of the relatively small size of our cohort, it was not possible to correct for more possible confounders than we already did. We therefore call for validation of our results in larger or multiple cohorts. Because of the low prevalence of VSCC, this can only be attained by multicenter and international collaboration.

Conclusion

Somatic mutations in *TP53*, *CDKN2A*(p16), and *HRAS* occur frequently in HPV-negative VSCCs. Patients with a somatic mutation, and especially those with a mutation in *HRAS*, have a significantly worse prognosis than patients without these changes.

Conflict of interest statement

None of the authors have any potential conflicting interests to declare.

Disclosure

This paper is original work and is not under consideration for publication elsewhere.

Part of these results have been presented at the 2013 Annual Meetings of the British Gynaecological Cancer Society and the European Society for Gynaecological Oncology.

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