Vulvar cancer : pathogenesis, molecular genetics and treatment

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CHAPTER 7

Genomic characterisation of vulvar (pre)cancers identifies distinct molecular subtypes with prognostic significance

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Clinical Cancer Research 2017;23:6781-6789

Abstract

Purpose: Vulvar cancer (VC) can be subclassified by human papillomavirus (HPV) status. HPV-negative VCs frequently harbor *TP53* mutations; however, in-depth analysis of other potential molecular genetic alterations is lacking. We comprehensively assessed somatic mutations in a large series of vulvar (pre)cancers.

Experimental Design: We performed targeted next-generation sequencing (17 genes), p53 immunohistochemistry and HPV testing on 36 VC and 82 precursors (sequencing cohort). Subsequently, the prognostic significance of the three subtypes identified in the sequencing cohort was assessed in a series of 236 VC patients (follow-up cohort).

Results: Frequent recurrent mutations were identified in HPV-negative vulvar (pre) cancers in *TP53* (42% and 68%), *NOTCH1* (28% and 41%), and *HRAS* (20% and 31%). Mutation frequency in HPV-positive vulvar (pre)cancers was significantly lower (P=0.001). Furthermore, a substantial subset of the HPV-negative precursors (35/60, 58.3%) and VC (10/29, 34.5%) were *TP53* wild-type (wt), suggesting a third, not-previously described, molecular subtype. Clinical outcomes in the three different subtypes (HPV+, HPV-/p53wt, HPV-/p53abn) were evaluated in a follow-up cohort consisting of 236 VC patients. Local recurrence rate was 5.3% for HPV+, 16.3% for HPV-/p53wt and 22.6% for HPV-/p53abn tumors (P=0.044). HPV positivity remained an independent prognostic factor for favorable outcome in the multivariable analysis (P=0.020).

Conclusions: HPV- and HPV+ vulvar (pre)cancers display striking differences in somatic mutation patterns. HPV-/p53wt VC appear to be a distinct clinicopathologic subgroup with frequent *NOTCH1* mutations. HPV+ VC have a significantly lower local recurrence rate, independent of clinicopathological variables, opening opportunities for reducing overtreatment in VC.

Introduction

Traditionally, vulvar cancers (VC), of which the majority consist of squamous cell carcinomas, are sub classified depending on the presence or absence of human papilloma virus (HPV). HPV-positive (HPV+) VCs (about 30% of cases) originate in high-grade squamous intra-epithelial lesions (HSIL), formerly referred to as vulvar intraepithelial neoplasia of usual type (uVIN) (1, 2). In Europe, approximately 80% of VCs are HPV negative (HPV-), occur in older women, and are frequently associated with lichen sclerosus (LS) (1, 3). This subtype has been shown to frequently harbour *TP53* mutations. Differentiated vulvar intraepithelial neoplasia (dVIN) has been suggested to be the precancerous lesion preceding this subtype (3). dVIN has a high rate of malignant progression, which is estimated to be as high as 80% (1, 4). Other poorly characterised but putative HPV- VC precursors are verruciform lichen simplex chronicus (VLSC) and vulvar acanthosis with altered differentiation (VAAD) (5).

Few studies have investigated genetic alterations in VCs and its precursor lesions beyond HPV status (6-11). These studies have analysed a limited selection of genes using Sanger sequencing or small panel hotspot approaches (8, 10, 11). Thus far, a more comprehensive assessment of molecular alterations in VC and its precursors is lacking (12). Recent large-scale next generation sequencing (NGS) projects on other tumor types have delivered novel insights with clinical relevance (13-17). For head and neck cancer (HNC), a cancer with many clinico-pathological similarities to VC, large-scale NGS studies (13, 14) have showed that HPV+ and HPV- are molecularly distinct subtypes. Furthermore, these studies have advanced our understanding by identifying novel findings, such as a high frequency of *NOTCH1* mutations in HPV- tumors (13, 14).

The aim of the current study was to characterize the molecular landscape of VCs and their precursor lesions. We have taken advantage of the resemblance between HNC and VC by designing a targeted NGS approach including genes in pathways that have proven to be relevant in HNC. Our NGS results suggest that VCs can be classified into three categories: HPV+, HPV- with a *TP53* mutation and HPV- with wild type *TP53*. To investigate the clinical significance of this categorization, we also examined the clinical behaviour of these groups in a large cohort of 236 patients with VC in whom clinical follow-up was available.

Materials and methods

Sequencing cohort

Tissue samples

VC samples and precursor lesions (dVIN, LS, VAAD and HSIL) were collected from the pathology department in the Leiden University Medical Center. Sample selection was based on original diagnosis described in the pathology report and on the size of the available samples. Non-squamous vulvar cancers were excluded. From eight VC patients, adjacent precursor lesions were available and from one VC patient, sequential biopsies were included in the sample collection. In order to enrich for HPV-independent precursor lesions, an additional nationwide search for the HPV-independent precursor lesion dVIN was performed through the "nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA)" (18). Sample collection was approved by the medical ethics committee of the LUMC (reference number B16.024). All haematoxylin-eosin-stained (H&E) slides were re-evaluated by an expert gynaecologic pathologist (TB) blinded to molecular or immunohistochemical results.

Development of the targeted vulvar NGS panel

A targeted NGS panel (the VC NGS panel) was designed using previously published data on somatic mutations in VC, its precursors (12) and in HNC (13, 14, 19-21). The panel consists of 176 amplicons covering 97% of the coding region of 17 genes with a role in critical cellular pathways, such as differentiation, proliferation and apoptosis. The selected genes are; *BRAF*, *CASP8*, *CDKN2A*, *EZH2*, *FAT1*, *HRAS*, *KMT2C*, *KMT2D, KRAS*, *NOTCH1*, *NOTCH2*, *PIK3CA*, *SYNE1*, *SYNE2*, *NSD1*, *TP53* (covering exon 2-12), *TP63*. The primer sequences were synthesized by Integrated DNA Technology (IDT, Leuven, Belgium) and are available upon request.

DNA extraction

H&E-slides were reviewed by an expert gynaecologic pathologist (TB) who annotated the area, enriching for lesional cells. Unstained 10 μm sections (4 when the tumor was larger than 1 cm and 8 when the tumor was smaller than 1 cm) were cut from FFPE tissue blocks and dried at 37˚C overnight. The sections were deparaffinized in xylol, rehydrated and stained with haematoxylin after which the tumor tissue was manually microdissected based upon the previous annotation on the H&E slide. When possible, associated normal tissue was microdissected separately. After proteinase K digestion overnight, DNA was extracted according to the manufacturers protocol (Nucleospin® DNA FFPE XS, Macherey-Nagel). The obtained DNA was quantified using the Qubit dsDNA broad range assay kit (Life Technologies, Gent, Belgium). A minimum of 50 ng DNA was necessary in order to perform the targeted next generation sequencing (NGS).

Library preparation and sequencing

For library construction 50 ng of DNA was amplified using the primer pool from the designed targeted NGS panel. The samples were barcoded with an adapter and a patient specific barcode in a second round of PCR. After each round of PCR, purification with AmpureXP beads took place. Final sample pooling was based upon the Cq-values acquired with quality PCR. After sample pooling, size selection was performed and final concentration measured with LabOnAChip. Next, emulsion PCR and loading of the chip on the Ion Chef System was done. Subsequently, targeted NGS was performed with the Ion Proton™ System according to the manufacturer's instructions.

Mutation calling

The generated reads were aligned to the human genome (hg19) using the Burrows-Wheeler aligner (BWA, version 0.7.5a) (22). SNP and indel calling was carried out using VarScan software (version v2.3.6) with the following arguments: minimum read depth = 50, minimum number of reads with the alternative allele = 2, minimum base quality = 20, minimum variant allele frequency = 0.10 and p-value <0.01.

Variants were functionally annotated using ANNOVAR (23). We then selected the ones more likely to have a deleterious effect, which was done by focusing on non-sense, frameshift variants and variants known to be of clinical significance or with a cadd_phred score higher 15. Variants with a population frequency higher than 1% in the 1000 Genomes project (24) were removed, since they are more likely to be germline. The called mutations were visually inspected using Integrative Genomics Viewer (IGV) software by LN and DR (http//www.broadinstitute.org.igv).

Follow up cohort

Follow-up data, HPV-status and p53 immunohistochemical data were available from a follow-up cohort consisting of 236 patients with VC. These patients were consecutively treated for primary VC in the LUMC (147 patients) and the Hospital Clinic de Barcelona (89 patients) between 1983 and 2012. A local recurrence was defined as a histologically confirmed recurrence within two years after primary treatment. In the LUMC cohort only recurrences on the ipsilateral side of the vulva were considered a local recurrence.

HPV analysis

DNA extracted from two 10-µm whole tissue sections was used for HPV analysis. To prevent contamination and to serve as a negative control sections of a paraffin block without tissue were cut before each tumor sample. We performed the SPF-10 PCR from the INNO-LiPA HPV Genotyping Extra Amp kit (Innogenetics, Gent, Belgium) according to manufactures protocol to investigate whether or not HPV was present. All blank paraffin sections were negative for HPV in the final PCR analysis. HPV+ cases were

further genotyped using a reverse hybridization line probe assay (LiPA; Innogenetics) through which 25 individual genotypes could be identified. Only samples infected with high-risk HPV were designated as HPV+.

P53 immunohistochemistry

P53 expression in the VC was evaluated by immunohistochemistry . Mutational data for comparison was limited to the sequencing cohort, and not available for the follow-up cohort. Sections of 4 μm thickness were cut from formalin-fixed paraffin-embedded specimen blocks and dried overnight at 37˚C. Tissue sections were stained as described previously (25) using a monoclonal mouse antibody to p53 (Thermo scientific; 1:2000 dilution; clone DO-7). Two pathologists (VS and TB) performed all the scoring and interpretations of the IHC stains. Consensus meetings were held for the samples that were interpreted differently. P53 staining on VC was scored as "wild type" (p53wt) when nuclei of tumor cells stained weak to moderately, comparable to adjacent normal epithelium. Three patterns of staining were defined as "p53 abnormal (p53abn)"; 1) strong overexpression of all tumor cells 2) overexpression in the invasive tumor front or the undifferentiated / non-keratinized basal and parabasal cells at the interface with stroma, regardless of the location of the nests within the tumor mass, 3) completely absent staining in the tumor cells, with positive internal control showing a wild type pattern (26).

Statistical analysis

Statistical analysis was performed with SPSS version 20.0. We divided the patient groups into HPV +, HPV- with a p53 wild type staining pattern (HPV-/p53wt) and HPV- with a p53-abnormal staining pattern (HPV-/p53abn). The chi-square test was used to compare baseline characteristics between groups. Kaplan Meier analysis was performed to estimate local recurrence risk and overall survival. Multivariable analysis was performed with the Cox proportional hazard model and included age, tumor size, depth of invasion and lymph node status.

Results

Sequencing cohort

Sample characteristics

166 samples were collected for genetic characterisation. It was possible to isolate at least 50 ng of DNA for evaluation with targeted NGS from 125 samples. After library preparation and sequencing a total of 119 samples could be analysed (Figure 1). One sample was excluded from the final results due to repeating outlier results. Pathology review (HE only) showed high concordance with local pathology; in 108/118 (92%).

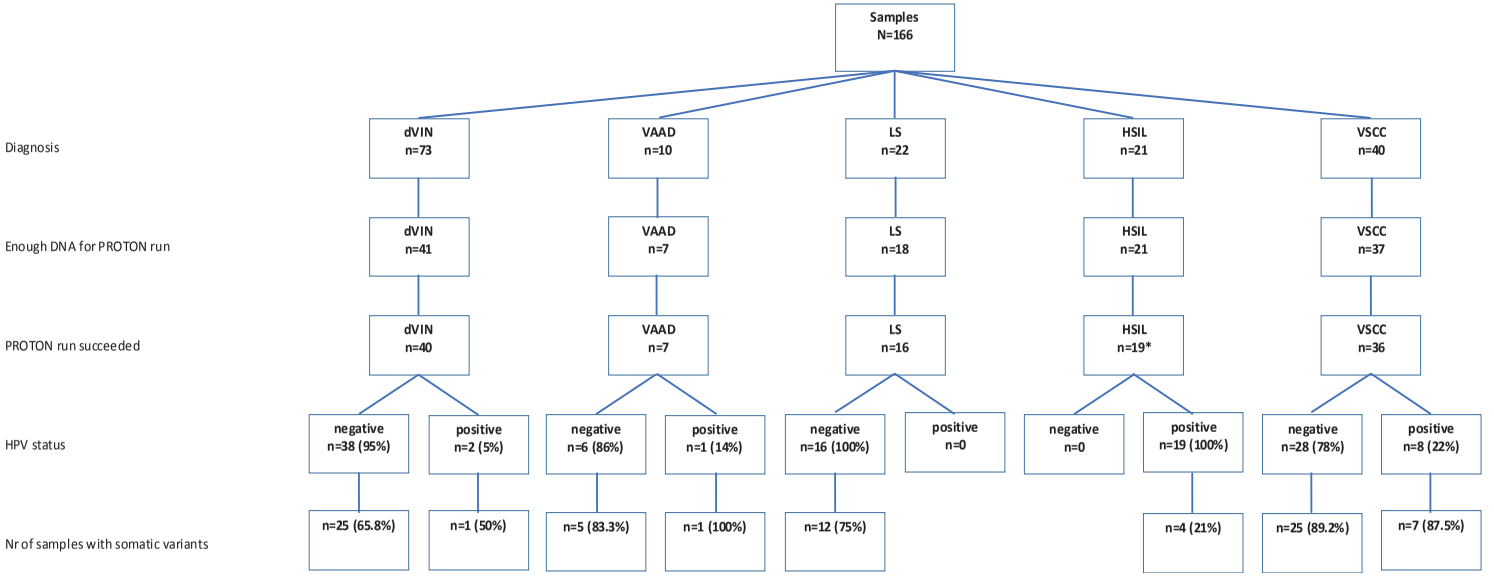

Figure 1: Flowchart sequencing cohort

dVIN: differentiated vulvar intraepithelial neoplasie
VAAD: vulvar acanthosis with altered differentiation
LS: lichen sclerosus
HSIL: high grade squamous intraepithelial lesion
VSCC: vulvar squamous cell carcinom
HPV: human papilloma virus
* one sample was excluded from the final analysis due to outlying results

Ten cases were discordant, of which eight dVIN (reclassified to VAAD (5 cases) or LS (3 cases)), one HSIL (reclassified to dVIN) and one LS (reclassified to VAAD). After revision the cohort for sequencing included 118 samples; 40 dVIN, 7 VAAD, 16 LS, 19 HSIL and 36 vulvar cancer samples. The diagnosis after review was used for all further analysis.

Sequence coverage

For the 118 samples analysed, the mean read length of each sequence was 162 bp and the average sequence was 147.7 Mb per sample. There was an average of 5177 reads per amplicon (range 0-496.101). 165/176 (93.8%) amplicons succeeded and 150/176 (85.2%) amplicons had an average of at least 50 reads. *CDKN2A* and *TP63* were removed from the final data analysis due to poor sequence coverage.

Mutational spectrum of precursor lesions and VC in relation to HPV status

The HPV status and somatic mutations found by NGS in relation to the histology are visually represented in a mutational heatmap in figures 2a (precursor lesions) and 2b (VC). Targeted NGS was performed and analysed on 82 precursor lesions; 22/82 (27%) were HPV+ and 60/82 (73%) were HPV-. Somatic mutations were significantly less frequent in HPV+ (7/22, 31.8%) than HPV- precursor lesions (43/60, 71.7%, p-value=0.001) (Figure 2a, table 1 and supplemental table 1). The most commonly mutated gene is *TP53* (26/82, 32% all sequenced precursor lesions; 1/22, 5% HPV+ and 25/60, 42% HPV-), followed by *NOTCH1* (20/82, 24% all sequenced precursor lesions; 3/22, 14% HPV+ and 17/60, 28% HPV-) and *HRAS* (15/82, 18% all sequenced precursor lesions; 3/22, 14% HPV+ and 12/60, 20% HPV-). In the HPV- precursor lesions 35/60 (58%) were *TP53* wild type. A somatic mutation in *NOTCH1* was found in 10/35 (29%) and in *HRAS* in 7/35 (20%) of these HPV-, *TP53* wild type precursor lesions. Most HPV+ cases were histologically classified as HSIL (19/22, 86%). Two of the HPV+ cases were diagnosed as dVIN. These cases retrospectively probably represent HSILs with superimposed inflammatory changes, mimicking dVIN. One HPV+ case was diagnosed as VAAD (2/40, 5% and 1/7, 14%, respectively). None of the LS cases included in this study was HPV+. Somatic mutations were significantly more common in the dVIN (65%), VAAD (86%) and LS (75%) samples, compared to HSIL (25%). Mutations in *TP53* were found in 19/40 (47.5%) dVIN, 1/7 (14.3%) VAAD, 5/16 LS (31.3%) and 2/19 HSIL (10.5%). Mutations in *NOTCH1* and *HRAS* were found in 8/40 (20%) and 4/40 (10%) dVIN, 2/7 (28.6%) and 5/7 (71.4%) VAAD, 8/16 (50%) and 5/16 (31.3%) LS and 2/19 (10.5%) and 1/19 (5.3%) HSIL samples, respectively. Finally, genes with lower mutational frequencies were *MLL2* (7/82, 8.5%), *MLL3* (5/82, 6.1%), *NSD1* (4/82, 4.9%), *NOTCH2* (4/82, 4.9%) and *SYNE1* (5/82, 6.1). Thirty-six VC samples were available for targeted NGS, 7 (19%) were HPV+ and 29 (81%) were

HPV-. Somatic mutations were found in 32/36 (89%) of all vulvar cancer samples (Figure 2b, table 1 and supplemental table 1). The frequency of somatic mutations was the same for HPV+ (6/7, 85.7%) as for HPV- (26/29, 89.7%) VCs. Multiple somatic mutations were less frequent in HPV+ than HPV- VCs (2/7, 28.5% HPV+ and 14/29, 48.2% HPV-). In the total cohort, most mutations were found in *TP53* (21/36, 58.3%). To investigate the potential utility of p53 immunohistochemistry as a surrogate marker for the identification of *TP53* mutational status, we performed p53-IHC on this sequencing cohort (supplemental figure 3). In 31/36 VC the results were concordant, resulting in a kappa of 0.72 (substantial agreement).

Other frequently mutated genes were *NOTCH1* (12/36, 33.3%) and *HRAS* (10/33, 27.8%). Finally, genes with lower mutational frequencies were *CASP8* (3/36, 8.3%), *MLL2* (3/36, 8.3%), *MLL3* (4/36, 11.1%), *NOTCH2* (2/36, 5.6%), *SYNE1* (5/36, 13.9%) and *SYNE2* (2/36, 5.6%). From eight VC patients included in our sequencing cohort adjacent precursor lesions (5 directly adjacent, and 3 distant but in same specimen) could be analysed. The results of these patients are shown in supplemental figure 1. An identical *TP53* mutation was identified in 3/5 precursor lesions directly adjacent to the VC. Interestingly, the somatic mutations in the remaining paired cases were distinctly different from the VC, suggesting that precursor lesions in these cases, despite the close proximity to the VCs, are likely unrelated (supplemental figure 1A).

From one patient interval biopsies and material from a tumor positive lymph node was available. This patient had VC on the right labium in 2001 which was treated surgically (no material available). In 2010 she had a local excision of a lesion from the left side of the vulva that showed a dVIN with possible micro-invasion (sample 1, supplemental figure 1B). In August 2011, she developed a dVIN lesion with possible micro-invasion on the right side of the vulva, which was surgically removed (sample 2). In October 2012, the patient developed VC in the midline of the vulva (sample 3). She underwent local excision combined with a resection of an enlarged left inguinal lymph node (sample 4). The same *NOTCH1* mutation was found in sample 2 and 3. Sample 3 and 4 contained the same mutation in *TP53*. These cases illustrate how the mutational profile may change during tumor progression.

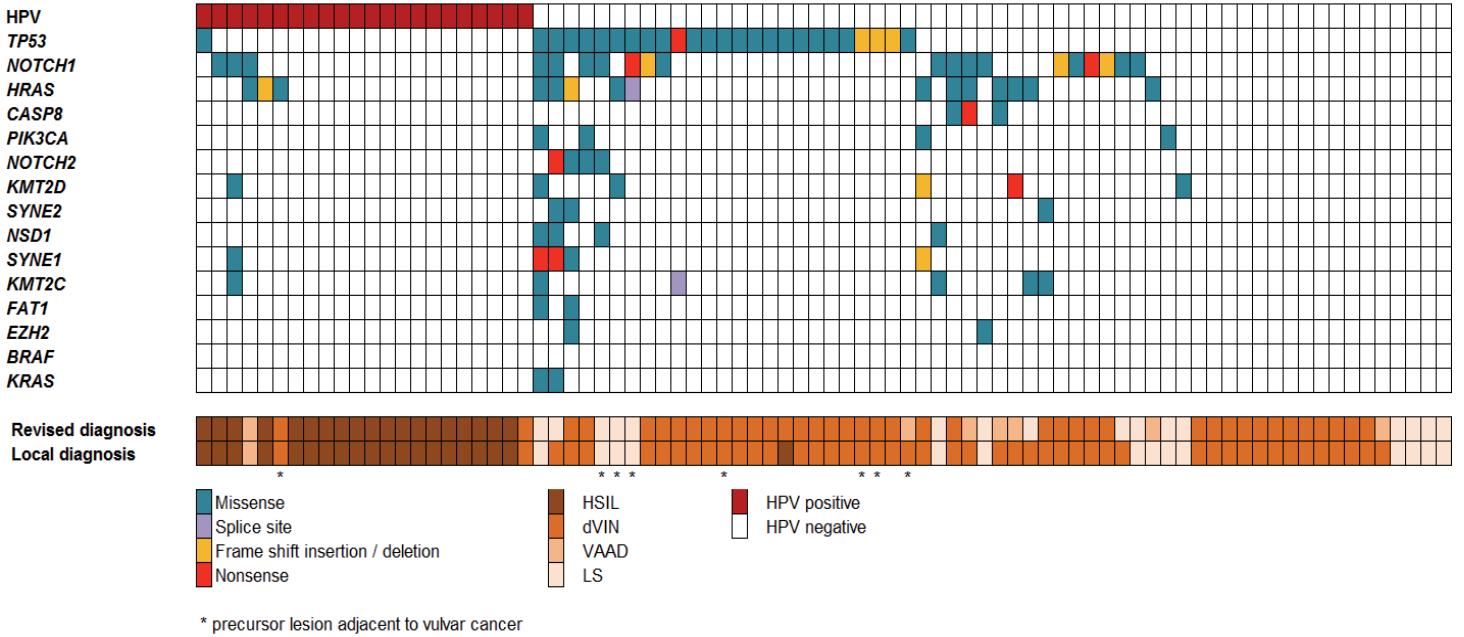

Figure 2a: Mutational spectrum of precursor lesions

HPV: human papillomavirus
HSIL: high grade squamous intraepithelial neoplasia
dVIN: differentiated vulvar intraepithelial neoplasia
LS: lichen sclerosis
*Precursor lesion adjacent to VC

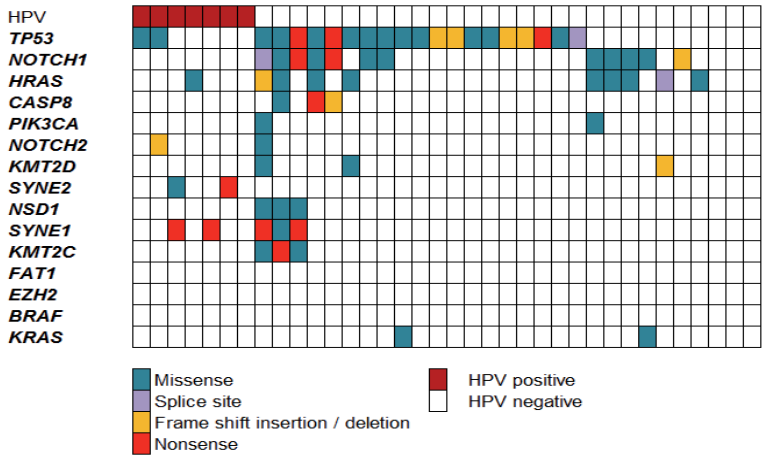

Figure 2b: mutational spectrum of vulvar cancers

Follow-up cohort

Prognostic implication of VC subtypes

Because our NGS results appear to suggest three distinct genetic subtypes of VC, 1) HPV+ VC, 2) HPV-/p53wt VC, and 3) HPV-/p53abn VC, we sought to determine the clinical outcome of this sub classification. For this, we analysed a second cohort of 236 VC patients for the presence of HPV and the expression of p53 by immunohistochemistry (follow-up cohort). Patient characteristics from the follow up cohort are described in table 2. HPV was positive in 38/236 (16.1%) patients and negative in 198/236 (83.9%) patients. In the HPV- group 43/198 (21.7%) had a wild type p53 expression pattern and 155/198 (78.2%) an abnormal p53 expression pattern. Two of the 38 patients with HPV+ tumors (5.3%) developed a local recurrence, whereas 7/43 (16.3%) of the patients with HPV-/p53wt tumors and 35/155 (22.6%) of the patients with HPV-/p53abn tumors developed a local recurrence (Figure 3a, p=0.044). The HPV+ patients were younger, had a lower FIGO stage and less often had tumor positive lymph nodes. When comparing the HPV-/p53wt and HPV-/p53abn groups with each other no clinical or tumor characteristics remained significantly different. There was no difference in local recurrence rate between the HPV-/p53wt and HPV-/p53abn groups (p=0.246). Five year survival was 75% for the patients with HPV+ tumors , 67.2% for the patients with HPV-/p53wt tumors and 56.3% for the patients with HPV-/P53abn tumors (supplemental figure 2, p=0.296). Disease specific survival was better for patients with HPV+ tumors compared to patients with HPV- tumors (Figure 3b, p=0.049). HPV+ status remained an independent favourable prognostic factor in multivariable analysis (Table 3, p=0.020).

Table 1: number of samples with somatic mutations found with the vulvar targeted next generation sequencing panel

	TP53	*BRAF*	*CASP8*	*EZH2*	*FAT1*	*HRAS*	*MLL2 (KMT2D)*	*MLL3 (KMT2C)*	*KRAS*	*NOTCH1*	*NOTCH2*	*NSD1*	*PIK3CA*	*SYNE1*	*SYNE2*
dVIN	19	0	1	1	1	4	2	1	0	8	2	0	2	2	2
(n=40)	47.5%	0%	2.5%	2.5%	2.5%	10%	5%	2.5%	0%	20%	5%	0%	5.0%	5%	5%
VAAD	1	0	2 28.6%	0	0	5	1	0	0	2	0	0	0	0	0
(n=7)	14.3%	0%		0%	0%	71.4%	14.3%	0%	0%	28.6%	0%	0%	0%	0%	0%
LS	5	0	0	1	1	5	3	3	2	8	2	4	2	2	1
(n=16)	31.3%	0%	0%	6.3%	6.3%	31.3%	18.8%	18.8%	12.5%	50%	12.5%	25%	12.5%	12.5%	6.3%
HSIL	1	0	0	0	0	1	1	1	0	2	0	0	0	1	0
(n=19)	5.3%	0%	0%	0%	0%	5.3%	5.3%	5.3%	0%	10.5%	0%	0%	0%	5.3%	0%
VC	21	0	3	0	0	10	3	4	2	12	2	3	2	5	2
(n=36)	58.3%	0%	8.3%	0%	0%	27.8%	8.3%	11.1%	5.6%	33.3%	5.6%	8.3%	5.6%	13.9%	5.6%
HPV-	19	0	3	0	0	9	3	4	2	12	1	3	2	3	0
(n=29)	65.5%	0%	10.3%	0%	0%	31.0%	10.3%	13.8%	6.9%	41.4%	3.4 %	10.3%	6.9%	10.3%	0%
HPV+	2	0	0	0	0	1	0	0	0	0	1	0	0	2	2
(n=7)	28.5%	0%	0%	0%	0%	14.3%	0%	0%	0%	0%	14.3%	0%	0%	28.5%	28.5%
P-value	*0.001*	*NA*	*0.024*	*0.522*	*0.522*	*0.001*	*0.500*	*0.227*	*0.142*	*0.071*	*0.520*	*0.005*	*0.520*	*0.522*	*0.823*

dVIN: differentiated vulvar intraepithelial neoplasia
VAAD: vulvar acanthosis with altered differentiation
LS: lichen sclerosis
HSIL: high grade squamous intraepithelial neoplasia
VC: vulvar cancer
HPV: human papillomavirus
NA: not available

Table 2: Patient characteristics (n=236)

	HPV+ (n=38)	HPV-/p53wt[1] (n=43)	HPV-/p53abn[2] (n=155)	P-value
Age (mean in years)	62 (25-92)	68 (36-93)	74 (37-96)	**<0.001**
FIGO 2009				**0.043**
Stage I	27 (71.1%)	28 (65.1%)	76 (49%)	
Stage II	4 (10.5%)	1 (2.3%)	10 (6.5%)	
Stage III	6 (15.8%)	13 (30.2%)	67 (43.2%)	
Stage IV	1 (2.6%)	1 (2.3%)	2 (1.3%)	
Tumor size				0.083
≤ 40 mm	30 (78.9%)	33 (76.8%)	103 (66.5%)	
> 40mm	5 (13.2%)	9 (20.9%)	47 (30.3%)	
Missing	3 (7.9%)	1 (2.3%)	5 (3.2%)	
Depth of invasion				0.253
≤ 4 mm	17 (44.7%)	17 (39.5%)	49 (31.6%)	
> 4mm	21 (55.3%)	26 (60.5%)	106 (68.4%)	
Primary treatment vulva				**<0.001**
Radical local excision	19 (50%)	26 (60.5%)	42 (27.1%)	
Vulvectomy	19 (50%)	17 (39.5%)	113 (72.9%)	
Tumor positive lymph nodes in the groin(s)	7 (18.4%)	15 (34.9%)	68 (45.6%)	**0.012**
Local recurrence	2 (5.3%)	7 (16.3%)	35 (22.6%)	**0.044**
Current patient status				**0.043**
Alive	26 (68.4%)	26 (60.5%)	84 (54.1%)	
Death	12 (21.6%)	17 (39.5%)	71 (45.9%)	
Median follow up time (months)	57 (2-174)	54 (6-172)	50 (0-206)	0.707

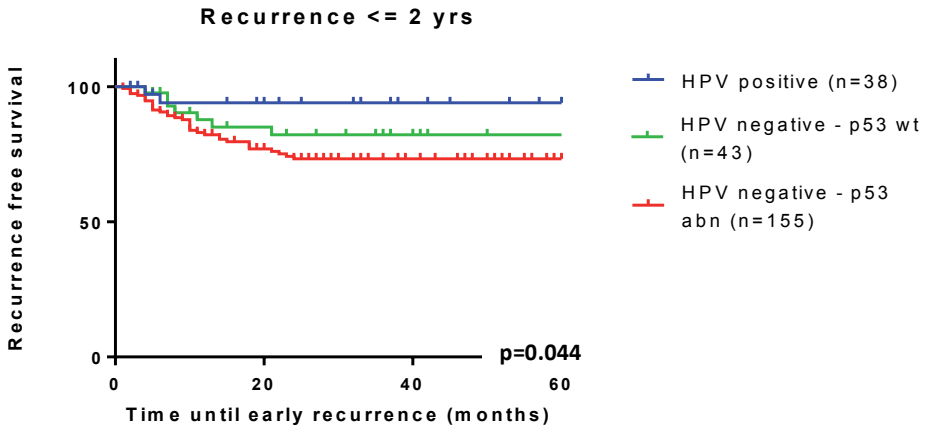

Figure 3a: Kaplan Meyer-curves for local recurrence rate

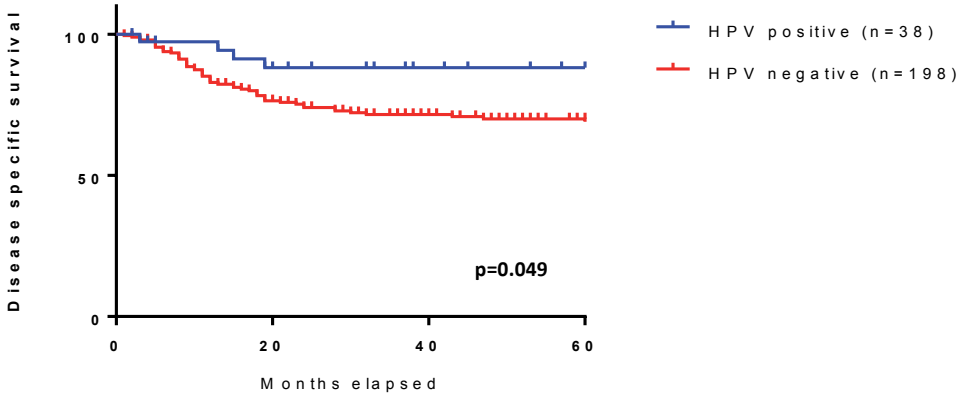

Figure 3b: Kaplan Meyer-curves for disease specific survival based on HPV status

Table 3: Multivariable analysis

Tumor characteristics	Hazard ratio (95% CI)	P-value
Age (mean in years)	1.024 (1.004 – 1.045)	**0.021**
Tumor size		
> 40 mm vs ≤ 40 mm	0.534 (0.291 – 0.981)	**0.043**
Depth of invasion		
> 4 mm vs ≤ 4 mm	2.077 (1.174 – 3.675)	**0.012**
Lymph node status		
Tumor positive yes vs no	1.119 (0.675 – 1.856)	0.663
HPV status		
Positive vs negative	0.287 (0.101 – 0.819)	**0.020**

Discussion

This is the first study using targeted NGS to describe the mutational landscape of vulvar precursor lesions (n=82) and VC (n=36). With this approach, we were able to describe a mutational landscape of vulvar precursor lesions and VC. We found frequent somatic mutations in *TP53, NOTCH1* and *HRAS* in HPV- precursors and VCs. This finding suggests a critical role for these genes in the early development of VC. This is the first report to identify frequent somatic mutations in *NOTCH1* in VC and its precursors. Mutations in *NOTCH1* co-occurred with *TP53* mutations, but were also identified in VCs and precursors that did not carry *TP53* mutations. The frequency of *NOTCH1* and/or *HRAS* mutations was the highest in the HPV- VCs without a *TP53* mutation (7/10, 70%) compared to the HPV- VCs with a *TP53* mutation (8/19, 42%) and HPV+ VCs (1/7, 14%). Similar differences in TP53 mutational rate between HPV- and HPV+ vulvar cancers were recently reported by Weberpals et al (27). In our analysis of the precursor lesions, a strikingly similar pattern was observed suggesting that *NOTCH1* and *HRAS* are likely drivers of vulvar carcinogenesis that can act independently of *TP53*. Therefore, these data support a third molecularly distinct subtype that is HPV independent and *TP53* wild type. In a large follow-up cohort of 236 patients with VC we were able to identify these three subtypes using straightforward, and clinically applicable methods. This approach resulted in significant differences in local recurrence rate in univariable (p=0.044) and multivariable analysis (p=0.020) for patients with HPV+ tumors compared to patients with HPV- tumors.

The finding of somatic mutations in *NOTCH1* (32/118, 27.1%) in VC and precursor lesions is a novel finding of the current study. Interestingly, two recent studies that performed whole-exome and whole-genome sequencing on HNC also identified

frequent *NOTCH1* mutations (13, 14). These studies focused on the genomic differences between HNCs with and without HPV, but did not stratify HPV-, *TP53* wild type from HPV-, *TP53* mutant HNCs. In light of our findings, we analysed these publicly available HNC data for the relation between HPV, *TP53* and *NOTCH1*. We found, similarly to our findings in VCs, that *NOTCH1* mutations in HNC are also predominantly found in HPV-, *TP53* wild type tumors (12/36, 33.3%) compared to HPV-, *TP53* mutant (37/185, 20%) or HPV+ (3/34, 8.8%, data not shown) (13). All these data strongly suggest that aberrant Notch signalling is involved in the carcinogenesis of a subset of HPV- squamous cell carcinomas from both vulvar and head and neck origin. Aberrant notch function has been found in many other tumor types (14, 28-31) and intriguingly is associated with both tumor suppressor as well as an oncogenic function (29, 32). Previous studies found a clear association in HNCs between inactivating mutations in *NOTCH1* and carcinogenesis. This indicates that notch has a primary tumor suppressor function in this tumor type and likely also in VC (14, 21). In line with this, prediction models indicated that most *NOTCH1* mutations identified in our NGS cohort are predicted to be inactivating (data not shown). Furthermore, aberrant notch signalling is proposed to be an early event in mouse models of oesophagus cancer (33). Interesting in this respect are our finding of several lichen sclerosus cases, carrying *NOTCH-1* mutations. Although we didn't have follow-up information on these cases, it is tempting to speculate *NOTCH-1* mutations may predict for progression. Interesting studies on targeted therapies of notch in solid tumors have evolved in the last years, however most often focussed on inhibition of notch signalling. Early-stage clinical trials are investigating inhibition of notch through inhibition of g-secretase (the enzyme responsible for cleavage of notch receptors and downstream signalling) as a potential anticancer therapeutic strategy (34). It will be worth to further investigate the exact role of *NOTCH1* in the carcinogenesis in vulvar cancer, as it might be a novel opportunity for targeted therapy. Another frequently mutated gene worthy of further exploration in VC, was *HRAS*. Previous work already identified somatic mutations in *HRAS* in HPV- VCs and showed an associated with a worse prognosis (6). *HRAS* is an oncogene involved in the RTK/RAS/PI(3)K pathway, and somatic mutations lead to cell proliferation (13, 14).

Previous studies have already noted upon the presence of HPV negative VC that are wild type for *TP53* (9, 10, 35-39). In the current study an in-depth genomic analysis of these VC further support the concept of this third molecular group in VCs. The finding that this third group is also present in HNCs and in vulvar precursor lesions favours this proposed 3-tiered classification. Molecularly this subgroup has the highest frequency of *NOTCH1* and *HRAS* mutations, but also other mutations in other genes were identified. Morphologically most of the HPV-, *TP53* wild type precursors were diagnosed as dVIN and VAAD. A recent report, supportive of our findings, also identified HPV-

and *TP53* wild type vulvar cancers and describe frequent activating *PIK3CA* mutations (73%) in this subset of cancers (40). We identified two *PIK3CA* mutations in our VCs (2/28, 7.1%). To further delineate the molecular characteristics of this HPV-, *TP53* wild type subgroup of VCs more in depth analysis, such as whole exome sequencing, will be required. In the follow-up cohort, the HPV-/p53wt group appeared to have an intermediate risk of recurrence, however this did not reach statistical significance when comparing this to the HPV-/p53abn group (p=0.264, data not shown). A possible explanation is that we were underpowered to detect an effect. Therefore, future larger studies will be required to establish whether the HPV-/p53wt VCs are not only a separate molecular group, but also clinically distinct.

Previous studies on the influence of HPV on prognosis in patients with VC found contradictory results. Some found no difference in local recurrence rate and overall survival (41-43), whereas others were able to find a prognostic benefit for HPV in univariate analysis (44-46). HPV remained a favourable prognosticator in multivariable analysis in only two other studies (45, 46). Although our study does not fulfil all criteria for a biomarker study (REMARK criteria) (47), it is the largest series of VC patients to date and shows the prognostic benefit of HPV in multivariable analysis. This finding is supported by a recently published study by McAlpine et al, who found a superior progression free survival and disease specific survival for patients with HPV+ VC in a cohort treated after 1995. Taken together the results of these studies, we can now put this discussion to rest and can conclude the HPV+ VC have a significant better clinical behaviour. Next, we need to discuss whether these finding should have consequences for the treatment of patients with VC. Interestingly, McAlpine et al noted that HPV status in a cohort treated before 1995, did not show a difference in outcome. This may suggest that the more conservative surgical approach that has been developed in the course of the years has led to worse outcomes for patients with HPV- VC (48). Currently, all patients with VC, irrespective of HPV status, are treated similarly, with surgery being the first choice of treatment (49). The outcome of the present study raises the question whether HPV testing (or p16 IHC, as an excellent surrogate (48)) should be performed on all VC biopsies to identify patients with HPV- tumors with a high risk of recurrence. A possible clinical implication might be to perform a more radical surgical procedure when HPV is not detected followed by a more stringent follow-up scheme due to the higher chance of developing a local recurrence. Furthermore, HPV status might also be utilized as a predictive marker for response to adjuvant treatment (48, 50). For HNC, where the favourable prognosis of HPV+ cancers also has been established, (51, 52) prospective studies are ongoing to investigate whether adjuvant chemotherapy can be omitted in HPV+ tumors (53, 54).

Of course, also this study has its limitations, our targeted NGS design relies on the parallels between VC and HNC. This leads to a directed search for somatic mutations but of course limits the discovery of novel gene mutations. Additionally, we were dealing with small biopsies of vulvar precursor lesions, limiting the extend of the DNA analysis. Unfortunately, we had to remove *CDKN2A* from our panel due to poor sequencing coverage. We were therefore unable to report on the frequency of *CDKN2A* mutations. Finally, we were not able to associate our molecular findings of the precursor lesions to clinical follow-up. Future studies should be designed to determine the possible prognostic capacity of somatic mutations in the progression to VCs.

In conclusion, this report is the first to establish a genetic landscape of a large cohort of VCs and precursors using targeted NGS. We identified a distinct mutational profile in HPV+ VCs and give a molecular description of a group of HPV-independent VCs without *TP53* mutations. This third molecular subtype of VC shows a high frequency of *NOTCH1* and *HRAS* mutations and appear to have its own precancerous lesions, morphologically in the spectrum of dVIN and VAAD. Using a large cohort of patients with VC with long term follow-up, we were able to identify HPV as a significant favourable prognostic factor. P53 status seems to further refine local recurrence risk in the HPV-independent VCs. The recognition that VCs can be classified in at least three distinct molecular subgroups using clinically applicable markers represents a promise for risk stratification and opens opportunities for precision medicine for patients with vulvar cancer.

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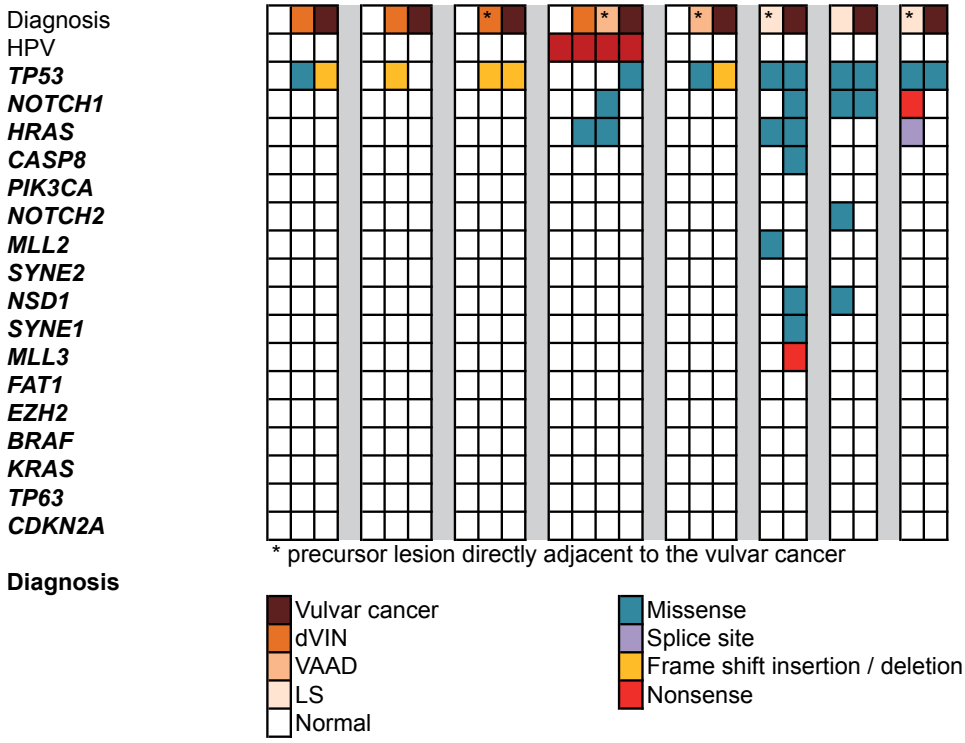
Supplementary data

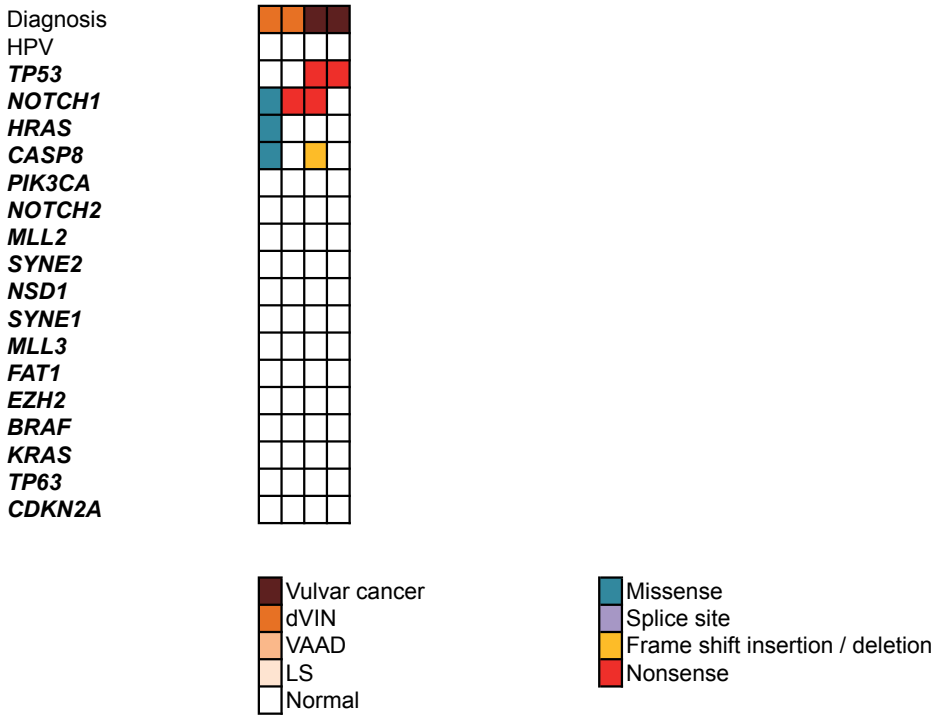
Supplemental table 1

Available upon request



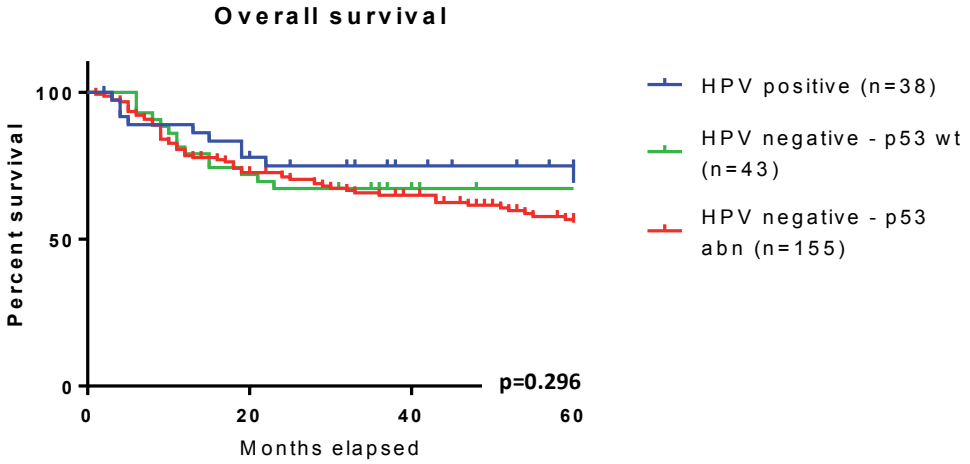
Supplemental figure 1a: Overview of somatic mutations found in normal and precursor lesion, adjacent to vulvar cancer

HPV: human papillomavirus
dVIN: differentiated vulvar intraepithelial neoplasia
VAAD: vulvar acanthosis with altered differentiation
LS: lichen sclerosus

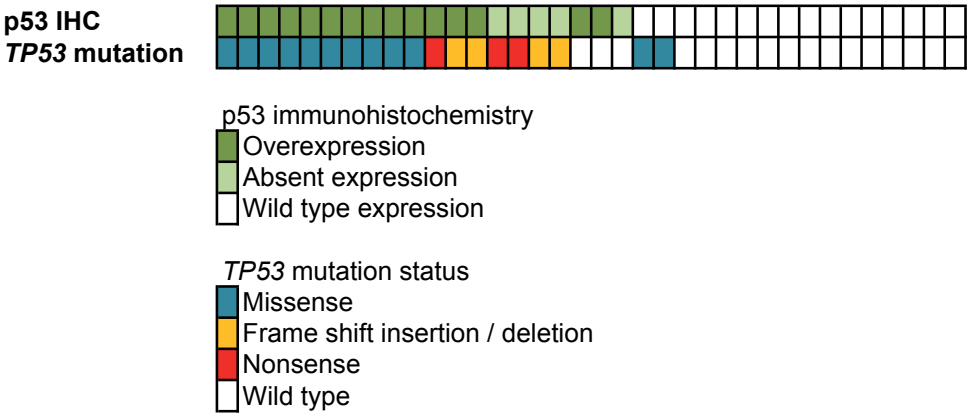


Supplemental figure 1b: Follow-up of one patient over time

HPV: human papillomavirus
dVIN: differentiated vulvar intraepithelial neoplasia
VAAD: vulvar acanthosis with altered differentiation
LS: lichen sclerosus



Supplemental figure 2: overall survival. HPV: human papillomavirus



Supplemental figure 3: overview of concordance between p53 immunohistochemistry and *TP53* mutation status in 36 vulvar cancer samples included in the sequencing cohort. Kappa = 0.72