Vulvar squamous cell carcinoma : genetics, morphology and clinical behaviour

Trietsch, M.D.

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Proefschrift
ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 9 november 2017
klokke 16.15

door

Marjolijn Dorothea Trietsch

Geboren te 's Gravenhage
in 1983

Promotiecommissie

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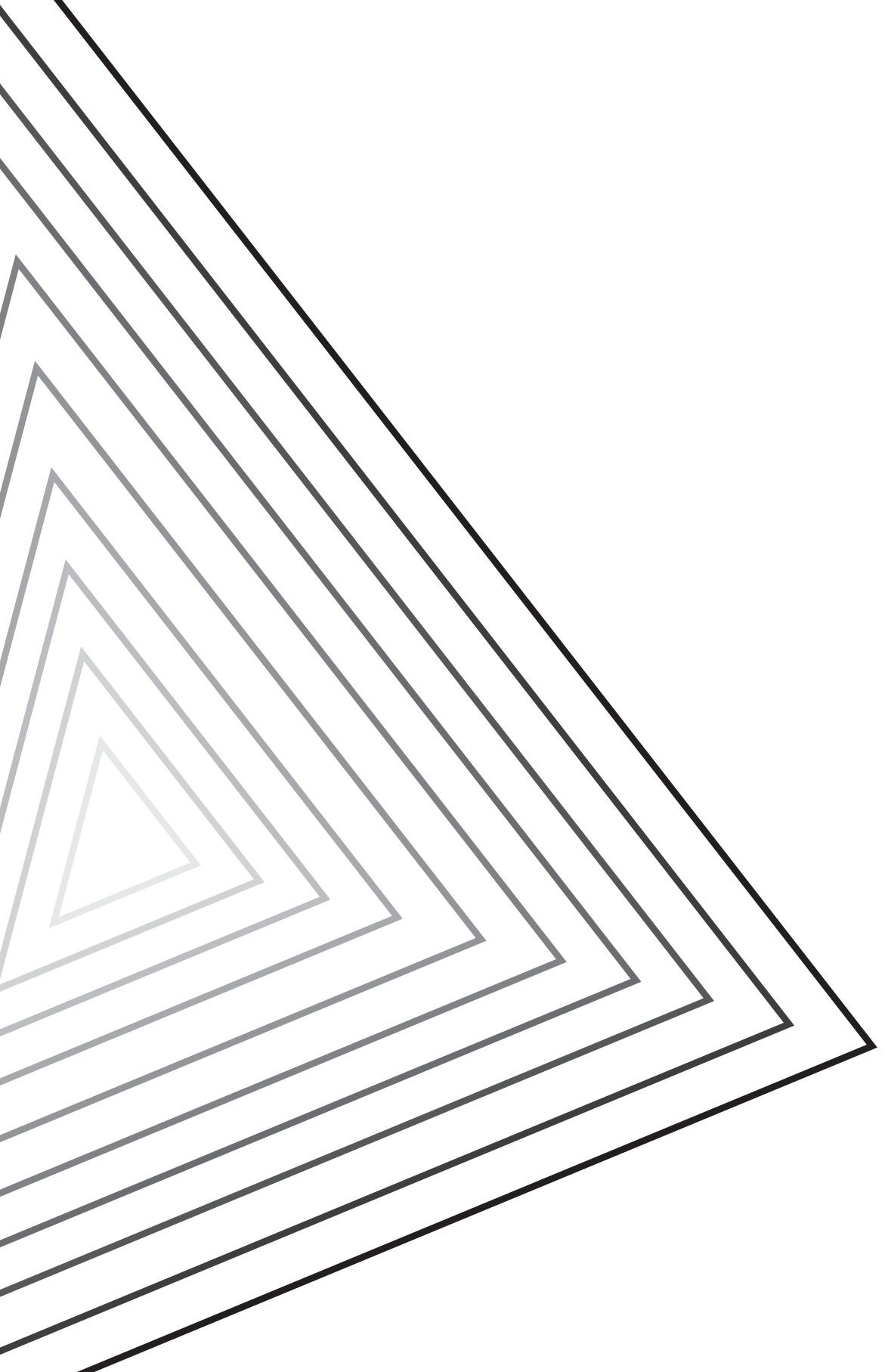
Vulvar squamous cell carcinoma – Genetics, morphology and clinical behaviour

The research described in this thesis was performed at the department of Pathology and the department of Gynaecology at the Leiden University Medical Center, Leiden, the Netherlands.

ISBN: 978-94-6233-769-5

Cover: Esmee Gramberg at AnnaGee Creative Studio (www.annagee.nl)
Layout: Rachel van Esschoten, DivingDuck Design (www.divingduckdesing.nl)
Printed by: Gildeprint Drukkerijen, Enschede (www.gildeprint.nl)

Content



Chapter 1

General introduction

1

General introduction

Although vulvar cancer is a rare gynaecologic malignancy with 2 new cases per 100,000 women each year, its impact on the often older aged patients can be devastating (1-7). When detected at an early stage, vulvar cancer can be treated curatively by surgical excision (8). Some patients however, suffer from rapid recurrences and progression (9). Vulvar cancer that has spread to the urethra, anus or lymph nodes requires larger or wider surgery, or a combination of surgery and radio- and chemotherapy, which in turn results in higher morbidity rates. This sometimes mutilating treatment has a major impact on the quality of life, because patients suffer from wound healing problems, lymph oedema and nerve damage that can result in sexual dysfunction and incontinence (10-12).

Currently, the aetiology of vulvar cancer has been far from unravelled. The majority of vulvar cancers are squamous cell carcinomas. It is well accepted that approximately 40% of all new cases of vulvar squamous cell carcinomas (VSCCs) are associated with a persistent infection by a high risk variant of the human papilloma virus (HPV) and that the other 60% are HPV negative and associated with mutations in the *TP53* gene (3;13;14). The HPV positive vulvar cancers are preceded by usual vulvar intraepithelial neoplasia (uVIN), a condition that has a malignant potential of 9-16% in untreated women (1;15) (figure met HE coupe). This type of vulvar cancer affects younger women and is associated with smoking and a higher number of sexual partners (1;3;16).

Differentiated vulvar intraepithelial neoplasia (dVIN) is the precursor lesion of the non HPV related vulvar squamous cell carcinoma (figure 1). This type of VIN is very hard to recognise and is mainly seen adjacent to vulvar cancer. Whether this means that it is a precursor lesion that rapidly progresses to cancer before it can be detected as a solitary lesion is still being debated (17;18).

Markers that can predict patient outcome other than lymph node metastases have not been established yet (19;20). This thesis contains work to further understand and predict why vulvar cancer can behave as aggressively as it sometimes does.

Treatment of vulvar cancer

The International Federation of Gynaecology and Obstetrics (FIGO) staging system uses clinical and histological parameters to subdivide patients into risk categories (table 1 FIGO staging) (21). Early stage vulvar cancer has a fairly good prognosis, but the prognosis rapidly declines with increasing stage (table 2 FIGO stage and survival) (22).

Surgical excision is the primary treatment for low stage vulvar cancer (12). Depending on the size and spread of the tumour, other treatment modalities come to play. In the Netherlands, patients with unifocal VSCC with a diameter <4 cm

Table 1 *FIGO staging in vulvar cancer:*

Stage	TNM Classification	Description
0	Tis N0 M0	Carcinoma in situ, intraepithelial carcinoma
I	T1 N0 M0	Confined to the vulva or perineum; no nodal metastasis
A	T1a N0 M0	Lesions =< 2 cm with stromal invasion, =< 1 mm
B	T1b N0 M0	Lesions > 2 cm in size or stromal invasion, > 1 mm
II	T2 N0 M0	Adjacent spread to the lower urethra, the vagina, or the anus, no nodal metastasis
III	T1,2 N1a,b N2a,b,c M0	Tumour confined to vulva or adjacent spread to the lower urethra, the vagina, or the anus and positive inguinofemoral lymph nodes
A	T1,2 N1a,b M0	One lymph node metastasis >= 5mm or 1-2 lymph node metastases < 5 mm
B	T1,2 N2a,b M0	Three or more lymph nodes < 5mm or 2 or more lymph nodes >= 5mm
C	T1,2 N2c M0	Lymph nodes with extracapsular spread
IV A	T1,2 N3 M0	Tumour with fixed or ulcerated lymph nodes
	T3 anyN M0	Tumour with spread into upper urethra/vagina, bladder, rectal mucosa, bone or fixed to pelvic bone
IV B	Any T Any N M1	Any distant metastasis, including pelvic lymph nodes

(FIGO 2009 and UICC 7th ed.)

Table 2 Five year survival according to FIGO Annual Report 2006, using the 1995 FIGO staging system:

Stage I: 79%
Stage II: 59%
Stage III: 43%
Stage IV: 13%

without suspicious groin nodes are generally treated with radical local excision and a sentinel node procedure (23). Tumours with a diameter exceeding 4 cm or multifocal tumours are generally treated with radical local excision and unilateral or bilateral inguinofemoral lymphadenectomy. In patients with tumours in FIGO stage III or higher but with contraindications for extensive surgery, such as high age and comorbidity, radical local excision is performed without inguinofemoral lymphadenectomy, followed by (chemo)radiation.

All of these treatments can be mutilating and have high morbidity rates (10;11). Wound healing problems, lymph oedema, and sexual dysfunction regularly occur. These negative effects of treatment can have a major impact on the patient's quality of life (10).

Aetiology of vulvar squamous cell carcinoma: two different pathways

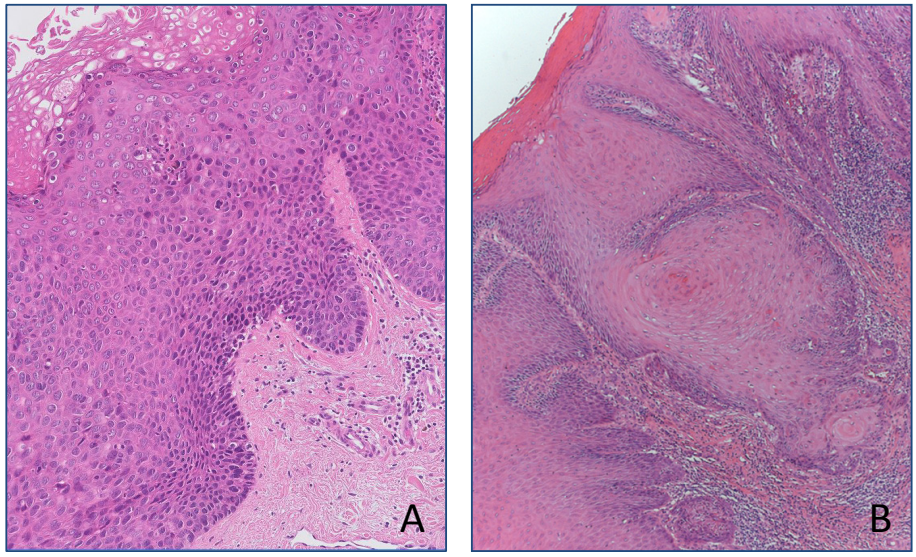
Cancer is the uncontrolled growth of abnormal cells, which can occur when the DNA in our cells is damaged and fails to be repaired. It can arise in virtually any cell type within the body. Skin cancer is one of the most prevalent types of cancer worldwide and is most often caused by DNA damage through ultraviolet irradiation

from sunlight. Well known skin cancer types are, amongst others, melanomas, basal cell carcinomas and squamous cell carcinomas.

The skin of the outer parts of the female genital tract, the vulva, can also be affected by cancer, but usually through a completely different etiologic pathway than in skin cancer on other locations of the body. As said before, most vulvar cancers are squamous cell carcinomas, a type of cancer arising from the squamous epithelium of the vulvar skin.

The pathogenesis of vulvar cancer can be divided into two different pathways: an HPV dependent, and an HPV independent route. Approximately 40% of all vulvar cancers are caused by a persistent infection with high risk HPV (3;13;14). HPV's are common and contagious viruses that can cause abnormal cell growth which can result in warts, and in some cases, cancer. More than 100 types of HPV have been described, of which 15 are 'high risk' types that can cause malignancies. These high risk HPV's are the main cause of cervical cancer and its precursor lesion cervical intra-epithelial neoplasia (CIN) (24). They can also cause the much less prevalent vulvar cancer and usual vulvar intraepithelial neoplasia (uVIN) (1;2;15;25). CIN usually goes unnoticed by its host, but VIN itself can be an unpleasant condition, resulting in itching and pain. Of VIN patients not receiving treatment, 9-16% will progress towards vulvar cancer (1;15). uVIN patients that do receive treatment have a risk of developing cancer from their VIN lesion of approximately 3% (figure 1a).

Figure 1 *Usual VIN and differentiated VIN*



HE slides of a usual VIN (A) and differentiated VIN (B). Note the uniform cells with hyperchromatic nuclei and little maturation in uVIN and the mature cells with eosinophilic cytoplasm in dVIN.

It is estimated that over 80% of women will have an HPV infection at least once in her life, whereas only a very small number of these women will suffer from cancer caused by HPV infection. In most cases, the HPV infection is transient, and the body is able to clear the virus by itself within 1 to 2 years (24). Some women, however, are unable to rid the virus, which finds a way to integrate itself in the human DNA and causes cell growth and, in some cases, eventually cancer. Smoking and a compromised immune system (for example by HIV/AIDS) are factors that have been proven to result in a higher risk of persistent HPV infection and therefore a higher risk of HPV associated cancer (24). Other than that, little is known about why some women are unable to clear the HPV infection.

The other 60% of vulvar cancers are not associated with HPV infections, but with lichen sclerosis, a chronic, autoimmune inflammatory disease of the skin. Lichen sclerosis progresses towards cancer in 3-5% of patients (1;15). This pathway has a worse prognosis than the HPV positive pathway, and affects older patients than the HPV positive pathway, with an average age at diagnosis of 70 versus 40 years (1-3;15;25). Approximately 3-5% of women with lichen sclerosis will develop vulvar squamous cell carcinoma. Differentiated VIN (dVIN) is thought to be the precursor lesion of HPV negative vulvar cancers, but it is hardly ever diagnosed before invasive cancer has developed. This is probably due to its subtle clinical and histological appearance, which makes it hard to recognise by clinicians and pathologists, but it could also mean that dVINs progress very rapidly into vulvar cancer (3;15) (figure 1b).

Somatic mutations in the HPV negative pathway

The HPV negative pathway is associated with lichen sclerosis, but also with somatic mutations. The most frequently mutated and well-studied gene in vulvar cancer, and in fact in any type of cancer, is the *TP53* (tumour protein 53) gene. This gene is the guardian of the cell cycle: it stops cells from dividing when aberrations in the DNA are detected. *TP53* malfunctioning because of a somatic mutation leads to an uncontrolled cell cycle and chromosomal instability, which can lead to the formation of tumours (26). Studies on somatic mutations in vulvar cancer other than in *TP53* are limited in number and size. Holway and Growden have found that quite a large percentage of the vulvar cancers and carcinomas in situ they studied carry mutations in *PTEN (*Phosphatase and tensin homolog), but the study sizes are rather small (27;28). O'Nions, Soufir and Gasco have reported mutations in *CDKN2A* (cyclin-dependent kinase inhibitor 2A) (29-31). Both *PTEN* and *CDKN2A* are also tumour suppressor genes that are involved in signalling pathways that control the cell cycle and stop cells from dividing or even cause cells to undergo apoptosis (32;33). Studies of melanoma, lung, colorectal, and breast carcinomas have shown that the somatic mutation status can be used to predict prognosis and guide tumour-spe-

cific treatment strategies (34-37). Clinical trials have shown promising outcomes of targeted therapies, such as in targeting the PI3K/AKT/mTOR pathway in colorectal cancer (35).

Epigenetic alterations

In addition to genetic mutations, in which the DNA sequence is changed, VSCC might also develop under influence of epigenetic changes. Epigenetic changes, such as hypermethylation, are heritable changes in gene expression without changes in the DNA sequence. Hypermethylation functions as a switch that can turn genes on or off, thus inactivating tumour suppressor genes (38-43). Hypermethylation of the promoters of *RASSF2A*, *MGMT,* and *TSP1* has been described in vulvar cancer (40).

Predictive morphological factors in vulvar cancer

As mentioned in the first part of the general introduction, clinical and histological parameters that constitute the FIGO staging system have been studied carefully and have proven their prognostic and clinical value in large cohorts of patients. Identifying more risk factors for poor survival is important to further reduce the mortality and morbidity of vulvar cancer patients, but few studies have found new factors of clinical importance (19). To date, lymph node involvement is the only accurate prognostic factor for survival and recurrence (20).

Recent publications have focussed on the potential prognostic value of morphological characteristics in vulvar squamous cell carcinoma (44). Vulvar squamous cell carcinomas with a spray patterned or spindle cell morphology are thought to represent a subgroup of vulvar cancers with a worse prognosis than 'conventional', solid vulvar cancers. Spindle cells are thin, slender, elongated cancer cells that infiltrate stromal tissue and that occur either as single cells or as cords of cells rather than as groups or islands. Spindle cell carcinoma is seen in many different types of cancer and is associated with a worse prognosis in cancers of the oral cavity, oesophagus and lung (45-49). Spindle-shaped epithelial cancer cells have lost their typical epithelial characteristics and seem to gain the ability to infiltrate the underlying stromal and lymphvascular tissue and bud from the primary tumour (50).

Epithelial to Mesenchymal Transition

Research by the Brazilian group of Rocha/AC Camargo Cancer Center (44) has pointed out that this invasive growth pattern in vulvar cancer might be caused by a process called Epithelial to Mesenchymal Transition (EMT). EMT is a process

that naturally occurs during embryonic development, but can also be seen during cancer progression (51). Cells undergoing EMT lose their epithelial characteristics, such as their cobble stone shape, cell-cell-adhesion and basal cell polarity, and gain mesenchymal traits that provide them with the capacity to migrate as single cells through the extracellular matrix (51). This way, EMT supports the process of solid tumours converting to invasive separate cells. Several signalling pathways control EMT, amongst others Wnt and NOTCH signalling. One of the major inducers of EMT is Transforming Growth Factor-β (TGF-β). In healthy cells, TGF-β has a tumour suppressor role, but in malignant cells, its role changes and TGF-β will induce cell motility and angiogenesis (52;53). Another cell motility promoter that is thought to be involved in EMT is L1-cell adhesion molecule (L1CAM), which is an emerging prognostic factor for metastasis in many cancer subtypes. L1CAM is a membrane glycoprotein involved in neural development where it has two roles: a cell adhesion function, and a cell motility promoting function. It is expressed by normal nerve tissue, but it is sometimes detected on tumour cell surface (54). The role and relevance of L1CAM has been studied in gynaecological cancers such as endometrial and ovarian cancer, but not yet in vulvar cancer.

Thesis outline

the etiology of HPV positive vulvar cancer is quite well understood, but the HPV independent axis remains to be unravelled. This thesis aims to gain knowledge on the origin of this type of vulvar cancer through the study of two mechanisms: genetic and morphological alterations in vulvar cancer.

In order to study somatic mutations in vulvar cancer, suitable techniques and methods had to be selected, that match the small numbers of patients, the relatively low quality and quantity of available DNA and the lack of prior knowledge we have from the available literature on somatic mutations in vulvar cancer other than *TP53*. **Chapter 2** describes the design of a somatic mutations profiling panel using mass spectrometry that is created especially for gynaecological cancers, focussing on the mutations that are most relevant in cervical, endometrial, ovarian and vulvar cancer. **Chapter 3** contains the results from applying this panel to a cohort of 108 vulvar cancer patients that were treated for primary VSCC in the Leiden University Medical Center between 2000 and 2009. In a review of the literature (**chapter 4**), these data are compared to the current knowledge on genetic and epigenetic changes in vulvar cancer and its precursors.

The majority of vulvar cancers are squamous cell carcinomas, but within this cancer type, morphological sub classifications can be made that can help us understand and predict the progression of vulvar cancers. **Chapter 5** describes a vulvar squamous cell carcinoma type called squamous cell carcinoma with spindle

cell morphology. This type of cancer progression is in some cases thought to develop through the process of EMT, which is reflected by the expression of EMT inducing molecule L1CAM. **Chapter 6** reports the prevalence and prognostic value of L1CAM expression in separate groups of vulvar cancer patients from the Leiden and the Groningen University Medical Centers. In **Chapter 7**, the relation between mutational and morphological data is studied by testing solid and spindle cell carcinomas and their recurrences and metastases for somatic mutations. **Chapter 8** gives an overview of the findings of this thesis and sheds some light on possible future research.

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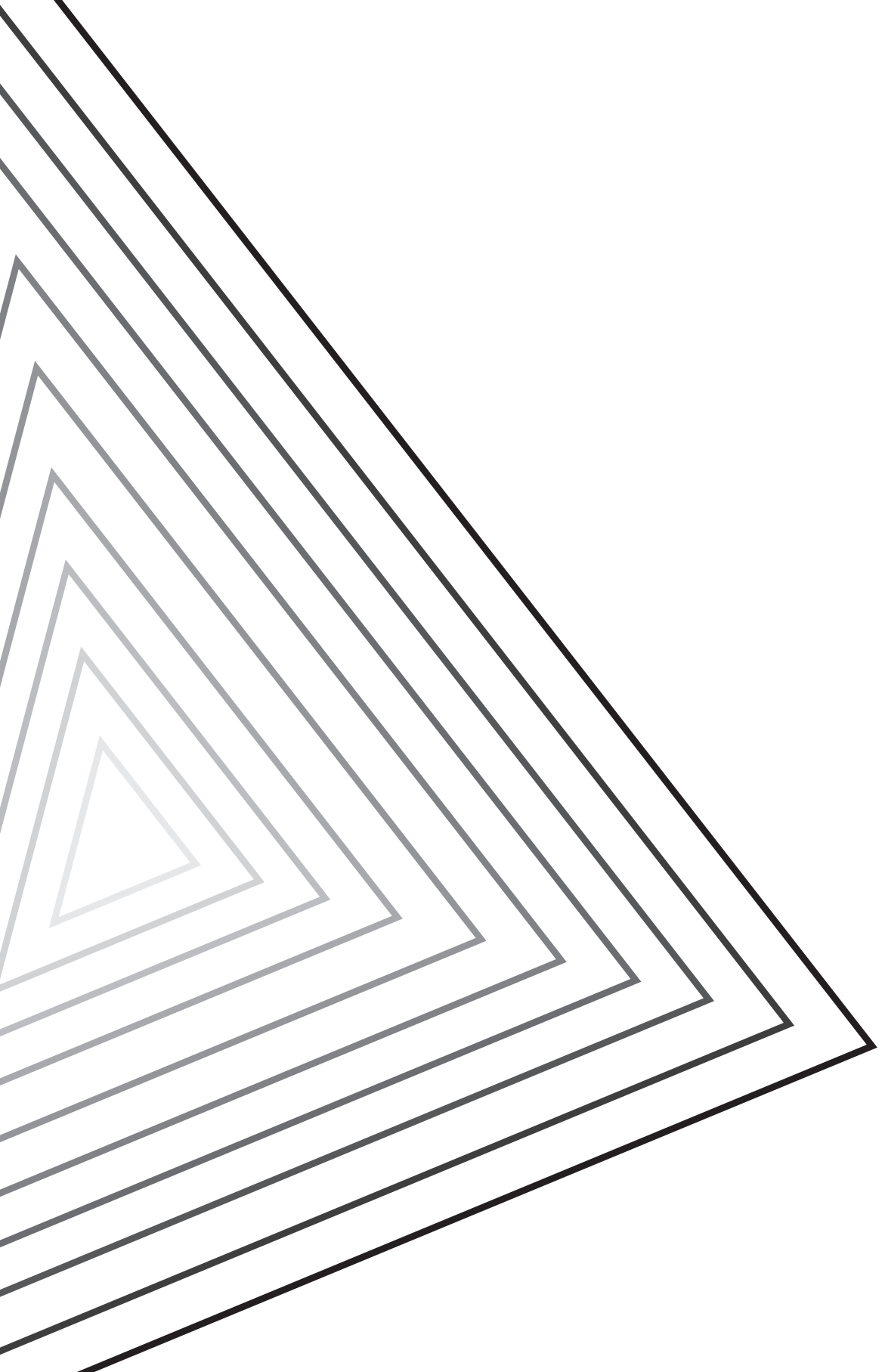

Chapter 2

Designing a high-throughput somatic mutation profiling panel specifically for gynaecological cancers

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PLoS ONE 9(3): e93451. doi:10.1371/journal.pone.0093451 2014

Abstract

Somatic mutations play a major role in tumour initiation and progression. The mutation status of a tumour may predict prognosis and guide targeted therapies. The majority of techniques to study oncogenic mutations require high quality and quantity DNA or are analytically challenging. Mass-spectrometry based mutation analysis however is a relatively simple and high-throughput method suitable for formalin-fixed, paraffin-embedded (FFPE) tumour material. Targeted gene panels using this technique have been developed for several types of cancer. These current cancer hotspot panels are not focussed on the genes that are most relevant in gynaecological cancers. In this study, we report the design and validation of a novel, mass-spectrometry based panel specifically for gynaecological malignancies and present the frequencies of detected mutations. Using frequency data from the online Catalogue of Somatic Mutations in Cancer, we selected 171 somatic hotspot mutations in the 13 most important genes for gynaecological cancers, being *BRAF, CDKN2A, CTNNB1, FBXW7, FGFR2, FGFR3, FOXL2, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A* and *PTEN*.

A total of 546 tumours (205 cervical, 227 endometrial, 89 ovarian, and 25 vulvar carcinomas) were used to test and validate our panel, and to study the prevalence and spectrum of somatic mutations in these types of cancer. The results were validated by testing duplicate samples and by allele-specific qPCR. The panel presented here using mass-spectrometry shows to be reproducible and high-throughput, and is usefull in FFPE material of low quality and quantity.

It provides new possibilities for studying large numbers of gynaecological tumour samples in daily practice, and could be useful in guided therapy selection.

Introduction

Cancer genomes carry somatic mutations, and the mutation spectrum varies by tumour type and subtype (1;2). Evaluating a broad range of key cancer gene mutations across diverse cancers has the potential for identifying clinically relevant mutations. Studies of melanoma, lung, colorectal, and breast carcinomas have shown that the somatic mutation status can be used to predict prognosis and guide tumour-specific treatment strategies (3-6). Gynaecological malignancies represent 15–20% of all new cancer cases in women worldwide, and numbers continue to increase (7), but the carcinogenesis of gynaecological malignancies is diverse and the role of somatic mutations is not yet fully elucidated (1).

Over the last decade, somatic mutations and their role in targeted therapy have been studied in gynaecological malignancies, but not yet to the same extent as in other types of cancer such as breast and colon cancer. Mutation profiling of gynaecological malignancies may identify novel drug targets and help predict patient prognosis and tumour response to treatment. Research has revealed overlapping genetic changes as well similar affected signalling pathways in the different types of gynaecological tumours (8-14).

When studying large numbers of patient material, we face two types of problems: technical applicability and tumour specificity. Nowadays, only a limited number of genes is screened in clinical practice. It is expected that this number will increase considerably in the near future. Therefore, a fast and trustworthy method to detect mutations is required. This technique must be suitable for DNA extracted from formalin fixed paraffin embedded (FFPE) tissue, which is often of low quality, or from small tissue biopsies, which is of low quantity. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) has proved to meet all these criteria (15-17).

As for tumour specificity, currently, several oncogene panels based on different techniques are (commercially) available. These panels have been successfully used in studying large amounts of tumour samples, in order to draw the landscapes of somatic mutations that characterise tumour types (18-22). A selection of genes and mutations relevant to tumour subtypes has successfully led to the design of tumour specific panels (15;16;23). As yet, there are no panels available that are specifically designed to target gynaecological tumours. Therefore, we aimed to develop a high-throughput mutation panel specified for gynaecological malignancies.

A meta-analysis of the COSMIC (Catalogue of Somatic Mutations in Cancer) online database (24), was performed to design a MALDI-TOF-based, high-throughput mutation panel that covers somatic mutations in 13 genes that are most frequently reported to be involved in gynaecological malignancies. We tested and validated this panel in a set of 546 cervical, endometrial, ovarian and vulvar carcinoma samples. Here, we present the design of a gynaecological cancer specific panel and the frequencies of somatic mutations identified using it.

Materials and methods

All human tissue samples in this study were used according to the medical ethical guidelines described in the Code for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences (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 receive information on the secondary use of tissue that is sampled for diagnostic use. They can actively object to secondary use. Accordingly to these guidelines, all human material used in this study has been anonymized. Because of this anonymization procedure, retrospective research does not require ethical approval from the Institutional Review Board and individual patients' permission is not needed.

Panel design

First, PubMed and COSMIC (24) searches were performed to select genes and mutations for inclusion in the gynaecologic-specific mutation panel. Selection was based on whether a mutation was repeatedly found to be mutated in gynaecological malignancies. Second, in order to cover a high percentage of the reported variants per gene, the most frequent mutations were selected to obtain a fair gynaecological-tissue-specific coverage, as only hotspot mutations were appropriate for analysis with the MALDI-TOF technique. We aimed to select genes in which for at least one of the studied gynaecological cancer types (e.g. vulvar, cervical, endometrial or ovarian cancer), at least 30% of all reported mutations occurred on less than 10 different sites on the gene

Establishing a gynaecologic specific 'hotspot' gene panel – GynCarta 1.0

Consulting PubMed and COSMIC databases clearly showed an overlap in top ten genes mutated in cervical, endometrial and ovarian cancer. Few somatic mutation studies have been performed on vulvar cancer and therefore for this tumour type we relied on frequencies found in similar tumour types (e.g. squamous cell carcinoma of the skin on other sites, and squamous cell carcinoma of head and neck). The most frequently mutated genes that met our inclusion criteria were selected for the panel. The first panel we designated 'GynCarta 1.0' (Sequenom®, Hamburg, Germany) consisted of 89 assays (12 multiplexes) to detect 154 mutations in 12 genes that met our inclusion criteria: *BRAF, CDKN2A, CTNNB1, FBXW7, FGFR2, FGFR3, FOXL2, HRAS, KRAS, NRAS, PIK3CA,* and *PTEN*.

Assay design

MySequenom.com online assay design tools were used to design the somatic mutation detection assays. A maximum multiplex level of 12 assays per well was applied. If possible, the mutant allele extension peaks were designed as first detected allele peaks and the wild-type extension peaks as the last detected allele peaks to reduce the danger of false positives from salt adducts. All assays were validated on wild-type DNA, negative controls and selected known positive mutation samples.

Mutation detection

Mutation detection was performed at the Leiden University Medical Center following the manufacturer's protocol (Sequenom®, Hamburg, Germany) as described previously [29]. Briefly, wild-type (WT) 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). Mutant and WT alleles were then discriminated using MALDI-TOF mass spectrometry.

Data analysis

Data were analysed with MassARRAY® Typer Analyser software (TYPER 4.0.22, Sequenom®, Hamburg, Germany). Mutations were detected by a minimum 5% threshold of the mutant allele peak. Three investigators blinded to tumour identification manually reviewed the output, and a consensus determination was reached. Statistical analyses were performed with IBM SPSS statistics Data Editor version 20.0. The independent Students *t*-test was used to compare baseline variables, and Fisher's exact test was used to analyse categorical and normally distributed numerical data. *P*-values ≤0.05, corresponding to 95% confidence intervals, were considered statistically significant. All tests were two-tailed.

Samples

First, a training set of 51 FFPE samples (26 cervical, 17 endometrial, 6 ovarian and 2 vulvar cancer samples) was used to test the efficacy of the designed panel. After minor technical adjustments and improvements of the panel, the number of patients for each tissue type was extended.

In total, DNA from 548 tumour samples from cervical (*N*=209), endometrial (*N*=227), ovarian (*N*=89), and vulvar (*N*=25) carcinoma patients was isolated. Two cervical cancer samples failed for all mass spectrometry assays and were excluded from further analyses. The following baseline parameters were collected: age, FIGO

(International Federation of Gynaecology and Obstetrics) stage, histopathological diagnosis, tumour grade if applicable, and human papillomavirus (HPV) positivity and type in cases of cervical and vulvar tumours (Table 1).

Table 1 *Baseline characteristics*

		Cervical carcinomas *N*=205	Endometrial carcinomas *N*=227	Ovarian carcinomas *N*=89	Vulvar carcinomas *N*=25
Age, median (IQR)		43 (35-55)	69 (65-75)	62 (52-69)	74 (52-80)
FIGO stage, *N* (%)	I	159 (78)	210 (92)	16 (18)	6 (24)
	II	44 (21)	11 (5)	13 (15)	8 (32)
	III		4 (2)	39 (44)	7 (28)
	IV			9 (10)	3 (12)
	Unstaged	2 (1)	2 (1)	12 (14)	1 (4)
Histology, *N* (%)	Squamous cell carcinoma	166 (81)			25 (100)
	Adenocarcinoma	24 (12)			
	Adenosquamous carcinoma	15 (7)			
	Endometrioid adenocarcinoma		206 (90)	42 (47)	
	Serous adenocarcinoma		17 (7)	26 (29)	
	Mucinous adenocarcinoma		2 (1)	13 (15)	
	Clear cell adenocarcinoma		2 (1)	6 (7)	
	Mixed-type carcinoma			2 (2)	
Grade, *N* (%)	1-2	N.A.	179 (79)	52 (58)	N.A.
	3		49 (21)	30 (34)	
HPV, *N* (%)	Positive	186 (91)	N.A.	N.A.	6 (24)
	HPV16	117 (63)			5 (83)
	HPV18	42 (23)			1 (17)

The baseline characteristics for all 546 gynaecological malignancies included in this study. IQR, inter-quartile range; FIGO, International Federation of Gynaecology and Obstetrics; HPV, human papillomavirus; N.A., not applicable.

DNA isolation

DNA was isolated from FFPE tissue blocks for 505 samples and from fresh frozen (FF) tissue for 43 ovarian carcinomas. Three to five 0.6-mm diameter tissue cores of variable length were taken from the FFPE blocks from a selected area comprising ~70% tumour cells. In 34 samples, tumour cells were diffusely distributed, and therefore micro-dissection was performed on 10 haematoxylin-stained 10-µm sections to obtain a high percentage of tumour cells. DNA isolation was performed as described before (25) followed by DNA purification (NucleoSpin® Tissue kit, Machery-Nagel, Germany) or was performed fully-automated using the Tissue Preparation System (Siemens Healthcare Diagnostics, NY, USA) [28].

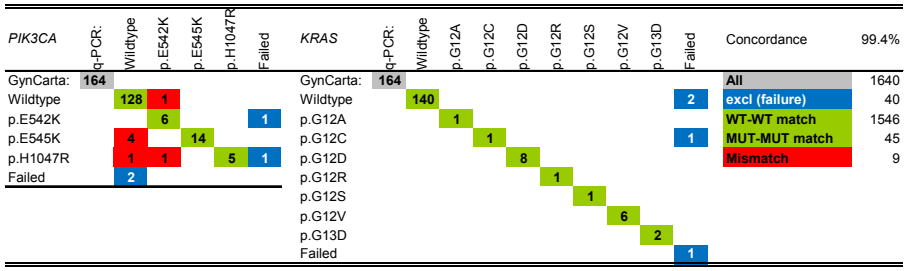
DNA quality

DNA of all samples was isolated and tested for quality; 493 (90%) samples scored ≥1 for DNA quality using PCR, and this was considered sufficient. Two samples, both cervical carcinomas with a DNA quality score of 0, failed in all assays, giving a success rate of 99.99%. Both samples were excluded from further analysis. In general, samples with low quality DNA were more likely to fail in some assays, but 27 out of 48 samples (56%) with DNA quality scores of 0 were analysed successfully in all assays, and 40 out of 48 (83%) low quality samples were analysed successfully in more than 90% of the assays. The percentage of successfully analysed samples did not differ significantly between FFPE and fresh frozen samples. This confirms that the MALDI-TOF mutation detection method is highly suitable for the analysis of lower quality, FFPE-extracted DNA.

Validation

In total, 546 tumour samples were included in this study. To assess assay reproducibility, 57 (10%) samples were tested in duplicate and another 26 (5%) in triplicate. Of the initially detected mutations in these samples, 95% (40/42) was confirmed in duplicate and 97% (30/31) was confirmed in triplicate. Non-template (*N*=4) and WT leukocyte DNA (*N*=2) controls were included in each multiplex to obtain negative and wild-type MALDI-TOF spectra. Furthermore, for a random30% (163 samples), *KRAS* and *PIK3CA* mutations were validated using allele-specific qPCR as described previously (26) on 7 mutation variants of *KRAS* (p.G12C, p.G12R, p.G12S, p.G12V, p.G12A, p.G12D, p.G13D) and 3 mutation variants of *PIK3CA* (p.E542K, p.E545K, p.H1047R), and a concordance rate of 99.4% was attained. (figure 1) The Gyn-

Figure 1 *Concordance between MALDI-TOF mutation genotyping and allele-specific qPCR results*



*The concordance between MALDI-TOF mutation genotyping (GynCarta, Sequenom®, Hamburg) and allele-specific qPCR for 3 PIK3CA and 7 KRAS mutations was determined for 164 (30% of the total cohort of 546 carcinomas) samples to validate the results. Concordance was calculated for all wild type-wild type matches (1546 in total) and all mutation-mutation matches (45 in total) in all reactions (164*10, 1640 in total). Failed reactions were excluded because comparison was not possible (4*3 for PIK3CA and 4*7 for KRAS; 40 in total). This lead to a concordance of (1546+45)/(1640-40) =0.994.*

Carta panel detected more mutations than allele specific qPCR did. This could be explained by the fact that mass spectrometry is able to detect mutant alleles with a lower frequency (down to 5%) than allele-specific PCR is (down to 20%). The fact that we did not find any mutations in the wild type control DNA, or in any of the H2O negative controls strengthens our belief that these additional mutations are true mutations rather than false positives.

Improving the panel and creating GynCarta 2.0

With the first mutational data from GynCarta 1.0 and literature reports of new oncogenic mutations, we were able to improve the GynCarta 1.0 panel by removing assays of mutations that were not detected (*CDKN2A* D108Y, D108XA, Y108XC; *FGFR3* Y373C, A391E, K650Q, K650E, K650T, K650M, S371C; *KRAS* G13S and *NRAS* G13V, G13A, G13D, G13C, G13R, G13S) and by adding 10 new hotspot mutations of the already included genes. This way, the coverage of *FGFR2* and *PIK3CA* was increased from 59% to 71% and from 72% to 76%, respectively. Furthermore, assays that had shown to be difficult to interpret because of small artefact peaks were improved. During the testing and validation period, *PPP2R1A*, a new gene of interest, had emerged from the literature (27-29). Nine mutations of this gene were also added to the panel, thus creating 'GynCarta version 2.0'. A complete overview of the mutations included in the GynCarta 2.0 mutation panel is given in table 2, with the added assays listed in bold. The assays for GynCarta 2.0 were organised in such a way, that a total of 13 multiplexes could be used to analyse the full panel, concentrating the new assays on 4 multiplexes. These 4 multiplexes were used to analyse the 497 samples of the confirmation set.

Results

Mutations identified using GynCarta 1.0 and 2.0

Mutation genotyping using GynCarta 1.0 revealed 395 mutations in 273 (50%) samples. The most mutations were detected in endometrial carcinomas (177 samples (64%)), followed by ovarian carcinomas (33 samples (37%)), cervical carcinomas (67 samples (33%)), and vulvar carcinomas (5 samples (20%)). *PIK3CA* was mutated most frequently (122 samples), followed by *PTEN* (97 samples) and *KRAS* (64 samples). No mutations were found in *BRAF* and *FOXL2*.

Mutation genotyping using GynCarta 2.0 detected an additional 36 mutations: 4 on *FGFR2* and 5 on *PIK3CA*. *PPP2R1A* mutations were detected in 27 samples (7 cervical, 18 endometrial, 2 ovarian and 0 vulvar samples).

Since panel version 1.0 and 2.0 had some overlapping assays, we were able

Table 2 *Design of GynCarta 2.0*

GENES (13)	*BRAF*	*CDKN2A*	*CTNNB1*	*FBXW7*	*FGFR2*	*FGFR3*	*FOXL2*	*HRAS*	*KRAS*	*NRAS*	*PIK3CA*	*PTEN*	*PPP2R1A*
Mutations	p.V600E	p.R58*	p.D32A	p.R465C	p.S252W	p.R248C	p.C134W	p.G12A	p.G12A	p.G12A	p.R88Q	p.K6fs*4	**p.P179L**
	p.V600K	p.R58X	p.D32G	p.R465H	**p.P253R**	p.S249C		p.G12C	p.G12C	p.G12C	p.E542K	p.E7*	**p.P179R**
	p.V600R	p.R80*	p.D32H	p.R479Q	**p.P253L**	p.G370C		p.G12D	p.G12D	p.G12D	p.E545A	p.F37S	**p.R183G**
	p.V600L	p.D108Y	p.D32N	p.R479L	p.Y375C	p.S371C		p.G12R	p.G12F	p.G12R	p.E545G	p.R84G	**p.R183W**
		p.D108A	p.D32V	p.R505C	**p.C382R**	p.Y373C		p.G12S	p.G12R	p.G12S	**p.E545D**	p.R130*	**p.R183Q**
		p.D108C	p.D32Y		**p.N549K**	p.A391E		p.G12V	p.G12S	p.G12V	p.E545K	p.R130fs*4	**p.S256F**
		p.W110*	p.S33A		**(T>A)**	p.K650E		p.G13C	p.G12V	p.G13A	p.Q546E	p.R130G	**p.S256Y**
		p.W110X	p.S33C		p.N549K	p.K650Q		p.G13D	p.G13A	p.G13C	p.Q546K	p.R130L	**p.W257C**
		p.P114L	p.S33F		(T>G)	p.G697C		p.G13R	p.G13C	p.G13D	**p.Q546R**	p.R130P	**p.R258H**
		p.P114X	p.S33P		**p.K659E**			p.G13S	p.G13D	p.G13R	**p.Q546P**	p.R130Q	
			p.S33Y					p.G13V	p.G13R	p.G13S	**p.Q546L**	p.R173C	
			p.G34E					p.G13X	p.G13V	p.G13V	p.Y1021C	p.R173H	
			p.G34R					p.Q61H	p.Q61E	p.Q61E	p.T1025A	p.Q214*	
			p.G34V					(C>A)	p.Q61H	p.Q61K	p.T1025X	p.R233*	
			p.S37A					p.Q61H2	(T>A)	p.Q61L	p.M1043I	p.R234W	
			p.S37C					(C>G)	p.Q61H	p.Q61P	(G>A)	p.P248fs*5	
			p.S37F					p.Q61K	(T>G)	p.Q61R	p.M1043I	p.C250fs*2	
			p.S37P					p.Q61L	p.Q61K		(G>T)	p.K267fs*9	
			p.S37T					p.Q61P	p.Q61L		**p.M1043V**	p.K267fs*31	
			p.S37Y					p.Q61R	p.Q61P		p.H1047L	p.V290fs*1	
			p.T41A						p.Q61R		p.H1047R	p.L318fs*2	
			p.T41I								p.H1047Y	p.T321fs*23	
			p.T41N									p.N323fs*2	
			p.T41S									p.N323fs*21	
			p.S45C									p.R335*	
			p.S45F										
			p.S45P										
			p.S45Y										
Total (171)	**4**	**10**	**28**	**5**	**6**	**9**	**1**	**18**	**19**	**17**	**20**	**25**	**9**
Assays (99)	**2**	**5**	**12**	**4**	**5**	**8**	**1**	**8**	**7**	**6**	**13**	**22**	**6**

The panel GynCarta 2.0, (Sequenom®, Hamburg) consists of 13 multiplexes containing 99 assays to detect 171 mutations in 13 genes that are most frequently described to be involved in gynaecological malignancies according to a COSMIC meta-analysis. Assays that were added to create GynCarta 2.0 are depicted in bold.

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Table 3 *Mutation Frequencies as detected by GynCarta 2.0*

Tissue Gene	CC[1] N=205	EC[2] N=227	OC[3] N=89	VC[4] N=25	Total N=546
PIK3CA[5]	**50 (24)**	**67 (30)**	**10 (11)**	**1 (4)**	**128 (23)**
p.E545K	33	13	1	1	48/542
p.H1047R	2	13	5	0	20/542
p.E542K	15	3	1	0	19/542
p.R88Q	1	16	1	0	18/542
p.M1043I(T)	0	5	1	0	6/535
p.Q546R	0	3	1	0	4/468
p.Y1021C	0	4	0	0	4/538
p.T1025A	0	4	0	0	4/530
p.H1047Y	0	3	0	0	3/541
p.E545A	0	2	0	0	2/542
p.Q546K	0	2	0	0	2/537
p.Q546L	0	1	0	0	1/468
p.M1043I(A)	0	1	0	0	1/535
p.M1043V	0	1	0	0	1/490
p.H1047L	0	1	0	0	1/542
PTEN[5]	**5 (2)**	**89 (39)**	**3 (3)**	**0**	**97 (18)**
p.R130G	1	35	1	0	37/542
p.R130fs*4	0	19	2	0	21/545
p.L318fs*2	0	10	0	0	10/542
p.R233*	0	7	0	0	7/543
p.R130*	1	5	0	0	6/542
p.T323fs*2	0	5	0	0	5/542
p.R173C	0	4	0	0	4/540
p.R173H	0	2	1	0	3/539
p.E7*	0	3	0	0	3/545
p.K267fs*31	1	2	0	0	3/542
p.R130L	0	1	0	0	1/544
p.R130P	0	1	0	0	1/544
p.R234W	1	1	0	0	2/495
p.K267fs*9	0	2	0	0	2/536
p.Q214*	1	1	0	0	2/544
p.P248fs*5	0	1	0	0	1/545
p.V290fs*1	0	1	0	0	1/542
KRAS	**9 (4)**	**39 (17)**	**16 (18)**	**0**	**64 (12)**
p.G12V	2	10	8	0	20/544
p.G12D	4	13	3	0	20/544
p.G13D	0	8	0	0	8/544
p.G12C	1	3	2	0	6/544
p.G12A	0	4	1	0	5/544
p.Q61H(G)	0	1	1	0	2/542
p.G12S	1	0	0	0	1/544
p.G12R	0	0	1	0	1/544
p.G13S	1	0	0	0	1/465

Tissue Gene	CC N=205	EC N=227	OC N=89	VC N=25	Total N=546
CTNNB1[5]	**7 (3)**	**33 (15)**	**3 (3)**	**0**	**43 (8)**
p.S37F	1	10	0	0	11/537
p.S45F	1	5	0	0	6/543
p.G34R	2	1	0	0	3/537
p.T41A	1	3	0	0	4/546
p.D32V	0	2	0	0	2/543
p.D32Y	0	2	0	0	2/543
p.S33F	0	2	0	0	2/542
p.D32N	1	1	0	0	2/543
p.S37C	0	1	1	0	2/537
p.T41I	1	0	0	0	1/542
p.S37P	0	1	0	0	1/544
p.D32H	0	1	0	0	1/543
p.S33A	0	1	0	0	1/544
p.S33C	0	1	0	0	1/542
p.S33Y	0	1	0	0	1/542
p.G34V	0	1	0	0	1/542
p.S45P	0	1	0	0	1/542
p.G34E	0	0	1	0	1/542
p.S37Y	0	0	1	0	1/537
PPP2R1A[5]	**7 (3)**	**18 (8)**	**2 (2)**	**0**	**27 (5)**
p.R258H	5	3	0	0	8/493
p.R183W	1	6	0	0	7/490
p.P179L	0	5	0	0	5/490
p.P179R	2	1	1	0	4/490
p.R183Q	0	2	0	0	2/463
p.S256F	0	1	1	0	2/463
FBXW7	**3 (1)**	**12 (5)**	**1 (1)**	**0**	**16 (3)**
p.R465H	2	6	1	0	9/536
p.R465C	1	3	0	0	4/540
p.R505C	0	3	0	0	3/542
FGFR2	**1 (<1)**	**13 (6)**	**1 (1)**	**0**	**15 (3)**
p.S252W	0	9	1	0	10/533
p.K659E	0	2	0	0	2/492
p.N549K (A)	1	1	0	0	2/491
p.N549K (G)	0	1	0	0	1/541
CDKN2A	**5 (2)**	**0**	**1 (1)**	**3 (12)**	**9 (2)**
p.R58*	3	0	0	1	4/535
p.R80*	0	0	0	2	2/535
p.W110*	1	0	1	0	2/541
p.P114L	1	0	0	0	1/540
NRAS	**1 (<1)**	**6 (3)**	**1 (1)**	**0**	**8 (1)**
p.G12S	0	2	0	0	2/542
p.Q61L	0	2	0	0	2/541
p.Q61K	0	1	1	0	2/541
p.Q61R	1	0	0	0	1/541
p.G12D	0	1	0	0	1/538
HRAS	**0**	**0**	**0**	**2 (8)**	**2 (<1)**
p.G12D	0	0	0	2	2/538
FGFR3	**1 (<1)**	**0**	**0**	**0**	**1 (<1)**
p.S249C	1	0	0	0	1/523
BRAF	**0**	**0**	**0**	**0**	**0**
FOXL2	**0**	**0**	**0**	**0**	**0**

Table 3 *Continued*

[1] Cervical, [2] endometrial, [3] ovarian, and [4] vulvar carcinomas. [5] 1 cervical and 5 endometrial samples had 2 PIK3CA mutations, and 11 endometrial samples had 2 PTEN mutations in the same tumour. One endometrial sample had 2 CTNNB1 mutations and 1 cervical sample had 2 PPP2R1A mutations in the same tumour. Frequencies presented as N(%), where N represents the number of samples showing the mutation. Mutations that were included in the panel but were not detected are not shown.

to compare the results of both panels. We did not detect any discrepant mutation calls, but we were able to analyse assays that were hard to interpret in GynCarta 1.0 because these assays had improved in GynCarta 2.0. We also obtained successful output for 3 samples that had failed in GynCarta 1.0. The mutation frequencies for each locus are summarized in table 3. The mutation spectrum is visualised in figure 2.

The detected mutation frequencies were compared with the predicted numbers of mutations based on the frequencies reported in the COSMIC database [23] and corrected for the panel coverage (Table 4). *PIK3CA* mutations were detected twice as frequently as predicted in cervical cancer (*N*=23 predicted and *N*=51 detected) and in endometrial cancer (*N*=32 predicted and *N*=71 detected). *PTEN* mutations were also detected more frequently in endometrial cancer than predicted (*N*=35 predicted and *N*=104 detected). However, no *PTEN* mutations were detected in vulvar cancer although *N*=8 mutations were predicted [19].

Furthermore, no *BRAF* or *FOXL2* mutations were detected in this cohort, despite the high coverage of both genes by the panel. This could be explained by the fact that *FOXL2* is strongly associated with granulosa cell tumours of the ovary (30), a subtype of ovarian cancer that was excluded from our study cohort.

GynCarta 2.0 can be used in differentiating tumour types

A visual illustration summarising the mutation frequencies in the different tumour types is depicted in figure 2. As shown in figure 2, gynaecological tumours show considerable overlap in somatic mutations, though tissue specific profiles can also be appreciated.

Endometrial cancers have the highest mutation frequency, with 78% of the samples carrying at least one mutation. As predicted, the most frequently mutated genes in gynaecological cancers are genes of the pAKT/mTOR pathway, but within this pathway, the mutational frequencies vary between tumour types. For ovarian cancer, *KRAS* is the most frequently mutated gene (18%), whereas *PIK3CA* is mostly affected in cervical cancer (24%) and *PTEN* in endometrial cancer (39%). Although the numbers of vulvar carcinomas included are small, vulvar cancer seems to have a different mutational spectrum as compared to other gynaecological malignancies with *CDKN2A* (12%) and *HRAS* (8%) most often affected.

An interesting difference can be observed when comparing *PIK3CA* distribution between cervical cancers and the other tumour types. In endometrial (and ovarian cancer), *PIK3CA* mutations are found most frequently on hotspots located on exon 9

Figure 2 *Mutation Spectrum*

COHORT	GENE	*N**	%	MUTATION SPECTRUM 25% 50% 75% 100%
Cervical cancer *N*=205	**Any Mutation**	**73**	**36%**	
	PIK3CA	50	24%	
	PTEN	5	2%	
	KRAS	9	4%	
	CTNNB1	7	3%	
	PPP2R1A	7	3%	
	FBXW7	3	1%	
	FGFR2	1	<1%	
	CDKN2A	5	2%	
	NRAS	1	<1%	
	HRAS	0		
	FGFR3	1	<1%	
Endometrial cancer *N*=227	**Any Mutation**	**178**	**78%**	
	PIK3CA	67	30%	
	PTEN	89	39%	
	KRAS	39	17%	
	CTNNB1	33	15%	
	PPP2R1A	18	8%	
	FBXW7	12	5%	
	FGFR2	13	6%	
	CDNK2A	0		
	NRAS	6	3%	
	HRAS	0		
	FGFR3	0		
Ovarian cancer *N=89*	**Any Mutation**	**33**	**37%**	
	PIK3CA	10	11%	
	PTEN	3	3%	
	KRAS	16	18%	
	CTNNB1	3	3%	
	PPP2R1A	2	2%	
	FBXW7	1	1%	
	FGFR2	1	1%	
	CDKN2A	1	1%	
	NRAS	1	1%	
	HRAS	0		
	FGFR3	0		
Vulvar cancer *N*=25	**Any Mutation**	**5**	**20%**	
	PIK3CA	1	4%	
	PTEN	0		
	KRAS	0		
	CTNNB1	0		
	PPP2R1A	0		
	FBXW7	0		
	FGFR2	0		
	CDKN2A	3	12%	
	NRAS	0		
	HRAS	2	8%	
	FGFR3	0		

The spectrum and frequencies of mutations identified using MALDI-TOF in 546 gynaecological carcinomas. The mutation spectrum is shown (from top to bottom) for cervical (N=205), endometrial (N=227), ovarian (N=89), and vulvar carcinomas (N=25). From left to right, N is the number of samples with the mutation, '%' is the percentage of mutated samples within the cohort, and bars represent the percentages graphically: blue, 4 mutations per sample (N=6); red, 3 mutations per sample (N=29); green, 2 mutations per sample (N=65); and yellow, 1 mutation per sample (N=189).

Table 4 *Coverage and frequencies of mutations in the gynaecologic-specific mutation panel*

	Cervical carcinoma *N*=205						Endometrial carcinoma *N*=227						Ovarian carcinoma *N*=89						Vulvar carcinoma *N*=25						Total Cohort *N*=546					
	COSMIC[1]		GynCarta 2.0				COSMIC[1]		GynCarta 2.0				COSMIC[1]		GynCarta 2.0				COSMIC[1]		GynCarta 2.0				COSMIC[1]		GynCarta 2.0			
	Frequency	Percentage	% Coverage	*N* expected	*N* detected	Percentage	Frequency	Percentage	% Coverage	*N* expected	*N* detected	Percentage	Frequency	Percentage	% Coverage	*N* expected	*N* detected	Percentage	Frequency	Percentage	% Coverage	*N* expected	*N* detected	Percentage	Frequency	Percentage	% Coverage	*N* expected	*N* detected	Percentage
BRAF	6/434	1	0	0	0	0	33/2254	1	26	1	0	0	253/3398	7	95	6	0	0	-	-	-	-	0	0	292/6086	5	86	7	0	0
CDKN2A	23/248	9	8	2	5	2	13/427	3	33	2	0	0	63/1378	5	13	1	1	1	1/27	4	100	1	3	12	100/2080	5	16	6	9	2
CTNNB1	7/130	5	57	6	7	3	283/1309	22	91	45	33	15	105/1521	7	86	5	3	3	-	-	-	-	0	0	395/2960	13	88	56	43	8
FBXW7	1/12	8	0	0	3	1	33/307	11	22	5	12	5	6/882	<1	33	0	1	1	-	-	-	-	0	0	40/1201	3	27	5	16	3
FGFR2	2/58	3	0	0	1	<1	88/927	9	77	16	13	6	4/857	<1	50	0	1	1	-	-	-	-	0	0	94/1842	5	71	16	15	3
FGFR3	6/414	1	83	2	1	<1	2/262	1	0	0	0	0	0/792	0	-	-	0	0	-	-	-	-	0	0	8/1468	<1	83	2	1	<1
FOXL2	0/28	0	-	-	0	0	0/216	0	-	-	0	0	329/1794	18	100	16	0	0	-	-	-	-	0	0	329/2038	16	100	16	0	0
HRAS	15/215	7	87	12	0	0	0/528	0	-	-	0	0	0/731	0	-	-	0	0	0/13	0	-	-	2	8	15/2487	1	87	12	2	<1
KRAS	45/617	7	100	14	9	4	327/2578	14	99	32	39	17	522/4203	12	99	11	16	18	0/14	0	-	-	0	0	894/7412	12	99	57	64	12
NRAS	2/127	2	100	4	1	<1	11/548	2	100	5	6	3	3/780	<1	100	0	1	1	0/13	0	-	-	0	0	16/1468	1	100	9	8	1
*PIK3CA**	39/332	12	94	23	51	24	562/2550	22	70	35	71	31	198/2366	8	85	6	10	11	-	-	-	-	1	4	799/5250	15	76	64	133	24
*PPP2R1A**	2/14	14	0	0	8	3	76/645	12	72	20	18	8	36/1354	3	84	2	2	1	-	-	-	-	0	0	114/2013	6	75	22	28	5
*PTEN**	20/406	5	29	3	5	2	824/2170	38	40	35	104	46	53/1487	4	40	1	4	3	5/8	63	54	8	0	0	906/4078	22	40	47	113	21

**The absolute number of mutations are reported; >1 mutation was detected in some tumours. COSMIC[1] database accessed February 2013. '-' indicates that data were not applicable.*

and exon 20, with an even distribution between these exons (33% and 45%). In cervical cancer however, mutations almost exclusively occur on loci on exon 9 (47 out of 50 (94%) *PIK3CA* mutations). This clear difference ($p<0.0001$) can be used in clinical practice, when differentiating primary cervical cancer from primary endometrial cancer.

Discussion

The demand for individualized cancer therapy has increased in recent years. New genotyping techniques allow tumours to be characterized based on their genomic profiles, which has revealed new targets for tumour-specific treatment, provided insights into tumour response to chemo- and radiotherapies, and helped predict patient outcome (3-6;9;12;14). Gynaecological malignancies account for 15–20% of all malignancies in women worldwide (7). The clinical consequences of somatic mutations in various gynaecological malignancies are not yet fully understood. In the present study, we designed a panel that is highly specific for a broad range of gynaecological cancers, to investigated the tumour-specific mutation spectrum of 162 mutations of 13 genes. Using this panel, we found that in this series somatic mutations were present in 36% of all cervical carcinomas, in 78% of endometrial carcinomas, in 37% of ovarian carcinomas and in 20% of vulvar carcinomas.

Somatic mutation spectra were investigated previously in gynaecological cancers also using MALDI-TOF (17;18;22;31-33). However, most of those studies used generic cancer gene panels based on the reported frequencies in all solid tumours or used pre-existing panels that were designed for general oncology (17;22;31-33). These pre-existing, commercially available panels are not adjusted to the field of gynaecological oncology, with the disadvantage of containing genes that are not involved in gynaecological cancers such as *FLT3* and *KIT*, or omitting genes that have shown to be involved relatively frequent in gynaecological cancer, such as *PIK3CA*. Therefore, we created a MALDI-TOF-based mutation panel designed specifically to detect a wide range of the most common hotspot mutations that have been reported in various types of gynaecological tumours. Similar mutation panels have been designed specifically for melanomas, colon carcinomas and non-small cell lung cancer (15;16;20). By using a gynaecological specific panel, we studied only relevant mutations, including for example *PIK3CA* and *PPP2R1A* that are not incorporated in general panels such as the OncoCarta (Sequenom, Hamburg, Germany) and with a better and more specific coverage (for e.g *CTNNB1*). As a result, the reported frequencies of gene-involvement can differ substantially. For example, in our series of endometrial cancer, a *KRAS* mutation rate of 17% was detected. This is in contrast to the study of Cote et al (32) that, using a generic onco-panel, reports a *KRAS* mutation rate of only 1% in endometrial cancer. From other studies using different techniques, it is known that *KRAS* is mutated in 15-20%

of all endometrial cancers (18;34), This example shows that the reliability of studies using a MALDI-TOF approach is seriously influenced by the choice and the extent of coverage of the genes incorporated in the panel.

Satisfactory coverage of the genes in our panel was achieved for the mutations we studied, and the mutation spectra generated in this study are thus a reliable representation of the mutation frequencies in gynaecological malignancies in the genes that are selected for this panel. However, some relevant genes, such as *TP53* and *ARID1a*, (8;13;34-39) were not included in our panel, because they did not fulfil the criterium of a "hotspot gene". Both genes have mutations scattered widely throughout the gene and were therefore not suited for a MALDI-TOF approach. There are some loci in *TP53* and *ARID1a* that are more frequently mutated; however these cover no more than 20% of all its known mutations. Including some of these loci in our panel would underestimate the true mutational frequency of these genes in gynaecologic cancers. Their mutation frequencies could be studied better using other detection methods, such as Sanger sequencing or by next generation sequencing (NGS).

We did decide to include 22 assays for the tumour suppressor gene *PTEN*, resulting in a 40% coverage, which could be considered suboptimal using this approach. The mutation frequency reported here is therefore likely underestimating the true somatic mutational frequency of *PTEN*. Additionally loss of PTEN can also be caused by other molecular alterations, such as LOH and promoter hypermethylation (40). Therefore, the additional use of other techniques such as immunohistochemistry is advised to evaluate the true status of PTEN.

CDKN2A is not truly a hotspot mutated gene too, but it was added to the panel because of its high predicted relevance in vulvar cancer, and because we expected to obtain a fair coverage of the gene. Tumours included in the COSMIC database frequently show complete loss of, or large deletions in the *CDKN2A* gene, a type of mutation that is not easily detectable by MALDI-TOF mass spectrometry. However, since *CDKN2A* mutations in squamous cell carcinoma of the skin are reported to be more often point mutations than (large) deletions, we believe that adding *CDKN2A* point mutations to the panel can give valuable information, especially for vulvar cancer. Although numbers are very low, results from research on *CDKN2A* imutations in vulvar and penile squamous cell carcinoma strengthen this hypothesis (41).

FBXW7 appears to have a low coverage by the panel, but this is influenced by the fact that it has been investigated and found to be mutated in relatively small numbers of gynaecological tumours. When considering the large numbers of available data from research in colon cancer, the expected coverage is approximately 35%.

Novel technologies such as next generation sequencing are able to detect mutations in multiple genes without preselecting and can therefore overcome the

limitations of a mass-spectrometry approach. With NGS, complete genes of interest can be analysed and therefore all mutations will be found. However, bioinformatic analysis of the data produced by NGS can be challenging and is currently still in development. Additionally, differentiating between non-pathogenic somatic variants and pathogenic mutations can be time consuming and complex (42). In comparison, MALDI-TOF data analysis is much more straightforward, particularly when analysing mutations with known clinical relevance. The panel we present here covers the most frequent mutations in gynaecological cancers, with a few exceptions. The mutation spectra we have detected are comparable to the spectra reported in NGS and exome sequencing studies that focus on gynaecological cancers (8;43;44). Therefore, MALDI-TOF mass-spectrometry has potential for use in a clinical setting, to detect the mutational status of relevant genes in a fast and reliable way. Another clear advantage of mass spectrometry based mutation analysis is the flexibility to add and delete assays from a panel, as also shown in this report, so new insights or clinical demands can be adopted easily.

The somatic mutation landscape of gynaecologic cancers produced by this study (figure 2) and by publicaly available mutation libraries show overlapping and distinguishing mutation profiles between gynaecologic tumours. Mutations in the PI3K/Akt-pathway are frequent and overlap, however some distinguishing mutations were identified. An example is the finding that *PIK3CA* exon 20 mutations only rarely occur in cervical cancer, whereas they are a frequent finding in endometrial cancers. This finding may be of value in a clinical setting, when there is uncertainty about the tumours primary origin, particularly in cervical adenocarcinomas that are HPV negative and located in the low uterine segment of the uterus. It illustrates that somatic mutational information may be useful for classifying tumours (45).

Somatic mutation profiling can also reveal new insights into tumour types that are not well characterised yet, such as vulvar cancer. Vulvar cancer is a rare disease that can arise through an HPV-dependent or an HPV-independent pathway. The carcinogenesis of HPV-independent vulvar carcinomas is largely unknown. In the present study, 25 vulvar carcinomas (of which 19 HPV-negative tumours) were analysed, and one *PIK3CA,* 3 *CDKN2A,* and 2 *HRAS* mutations were detected in the HPV-negative carcinomas. No mutations were detected in any of the 13 investigated genes in the 6 HPV-positive tumours. The mutation spectrum of vulvar cancer seems different from the spectrum of other gynaecological cancers, but shows similarities to the mutation spectrum of squamous cell carcinoma's of the head and neck (46), a tumour type that shares morphological and etiological characteristics with vulvar squamous cell carcinoma. The fact that vulvar cancer does not arise in Mullarian originated structures, as the other three tumour types in this study do, could also be an explanation for the differences in the spectrum that we have detected. The results of our study prompt further investigation of the roles of *HRAS* and *CDKN2A* in vulvar cancer.

In conclusion, we designed, validated and used a novel mass spectrometry-based mutation panel to identify somatic mutations in a large cohort of gynaecological malignancies. We have shown that this new panel is reproducible, high-throughput, and suitable for low quality and quantity DNA from FFPE samples. Our data support the potential for somatic mutation profiles as a tool to classify tumour types within the gynaecological tract. Furthermore, our results revealed that the PI3K-Akt signalling pathway is most prominently affected in gynaecological malignancies, justifying further investigation of *PI3K*/AKT/mTOR targeting therapies in gynaecological oncology. Future studies are required to determine whether this panel can be used to predict effective individualized, tumour-specific, and targeted treatment approaches.

Acknowledgements

We would like to thank Dr. E. S. Jordanova from the Department of Pathology for her valuable contributions to the design of this study and Prof. Dr. A. A. W. Peters, Dr. K. N. Gaarenstroom, Dr. M. I. E. van Poelgeest, and Dr. C. D. de Kroon from the Department of Gynaecology for their contributions to patient care and for their help in the registration of patient characteristics. We acknowledge Prof. Dr. C. L. Creutzberg and Dr. R. A. Nout from the Department of Clinical Oncology for help in providing the patient characteristics of the endometrial cancer patients.

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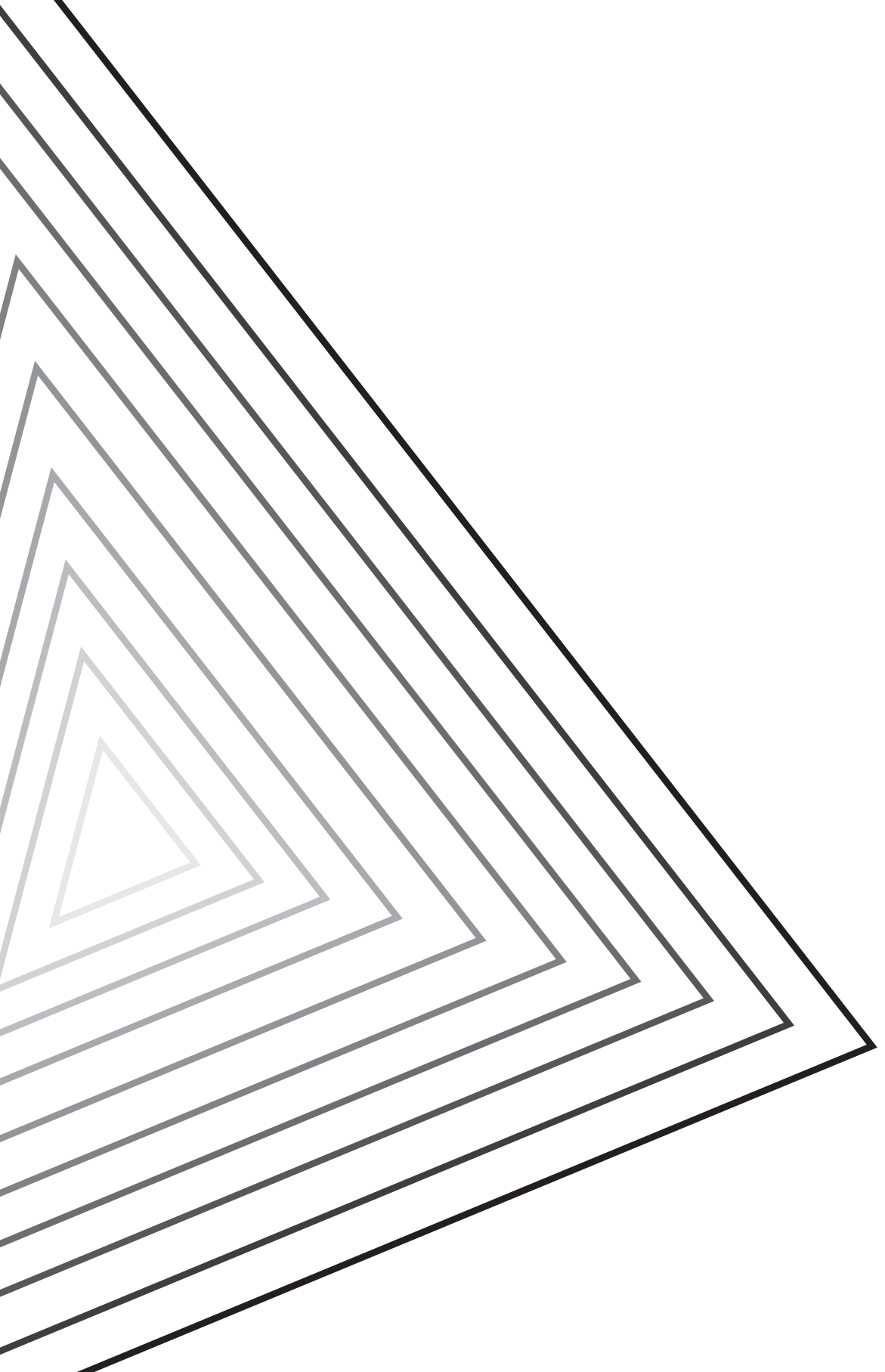

Chapter 3

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

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Gynecologic Oncology 135 (2014) 149–155

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 tumours (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.

Introduction

Vulvar cancer is the fourth most common gynaecological 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).

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 gynaecological 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 gynaecological cancer and SCC of the skin that could play a role in the tumourigenesis 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 centre for gynaecological cancers. Patients were excluded if they had received chemotherapy or radiotherapy in the pelvic area before surgery. Tumours from which only biopsies were taken were excluded from

the series. Tumour 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-99m nanocolloid combined with patent blue (17). Tumours with a diameter exceeding 4 cm or multifocal tumours were generally treated with RLE and unilateral or bilateral inguinofemoral lymphadenectomy. In patients with tumours 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)radiation.

Immunohistochemistry

Sections 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 tumour 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. Tumours 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% tumour 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 tumour 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 tumour 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.

Analysing *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 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 analysed with MassARRAY Typer Analyser software (TYPER 4.0.22, Sequenom, Hamburg, Germany) and MutationSurveyor (Softgenetics, State College, Pennsylvania, USA). Three investigators blinded to tumour identification inspected the cluster plots and mutation calls to discriminate true mutant calls from artefacts, 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 analyse 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, tumour size, and the most frequent mutations in this cohort were performed to analyse 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 tumour. Remaining recurrences were documented as distant recurrences.

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 tumour was taken and no further surgical treatment was given. Tumour tissue from one patient was of poor quality and was not analysed 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 harboured 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. Figure 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 tumour sample were significantly older (73 vs 65 years, $P = .002$) and had larger tumours (36 mm vs 26 mm, $P = .024$) with deeper infiltration of the tumour (7.5 mm vs 5.0 mm, $P= .003$). Tumour 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%, $P < .001$)

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

Characteristic			value	
Follow up†	– mo	(IQR)	38.0	(16.0-69.0)
Age at diagnosis‡	– year	(SD)	70.1	(14.0)
Duration of symptoms †	– 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)
Tumour size‡	– mm	(SD)	32.1	(22.2)
Infiltration depth†	– 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-yr overall survival	– %	(SD)	50.1	(5.1)
5-yr disease specific survival	– %	(SD)	67.8	(6.3)
5-yr disease free survival	– %	(SD)	27.9	(5.0)

† Median (interquartile range, IQR), ‡ mean (standard deviation, SD). Abbreviations: N = number; mo = months.

Survival Analysis

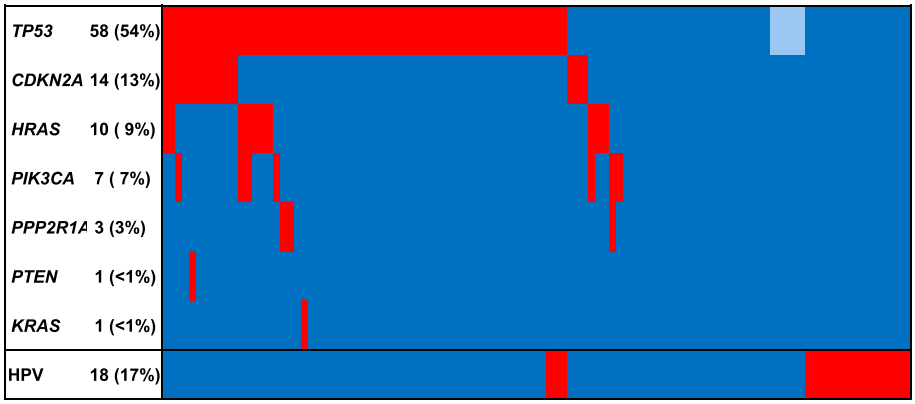
Patients carrying at least one mutation had a significantly worse overall 5-year survival (46.9% vs 58.9%, log-rank *P* = .035). Disease-specific 5-year survival was 67.8% in patients carrying a mutation versus 80.4% in patients without mutations (log rank *P* = .058) (Figure 2). Disease-free survival was comparable for patients with and without mutations (37.8% vs 43.6%, log rank *P* = .472). Cox regression analysis for disease

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)

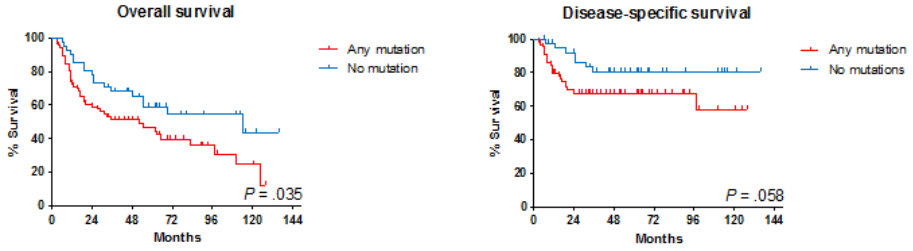
Abbreviations: hrHPV= high risk Human Papilloma Virus

Figure 1 *Mutation Spectrum*



Mutation spectrum for 107 VSCC patients. Blue = wild type or non-infected. Red = mutated or infected. Light blue = assay failed.

Figure 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 (blue) = no mutation detected; lower line (red) = mutation detected.

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†	– mo		34.5	(11.0-64.3)	55.0	(25.0-80.0)	0.043*
Age at diagnosis‡	– yr		73.4	(11.9)	64.9	(15.6)	0.002*
Duration of symptoms†	– 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*
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
Tumour size‡	– mm		36.0	(22.8)	26.1	(20.1)	0.024*
Infiltration depth†	– mm		7.5	(5.0-11.0)	5.0	(2.0-7.0)	0.003*
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*
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-yr Overall survival	– %	(SD)	46.9	(6.4)	58.9	(8.0)	0.035*
5-yr Disease specific survival	– %	(SD)	67.8	(6.0)	80.4	(6.7)	0.058
5-yr Disease free survival	– %	(SD)	37.8	(8.1)	43.6	(8.8)	0.472

**Significant difference (P < .05), †median (interquartile range, IQR), ‡mean (standard deviation, SD). Abbreviations: N = number; mo = months.*

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
Tumour 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

specific survival including age, HPV infection, tumour size, and the three most frequent mutations in this cohort (*TP53*, *CDKN2A*, 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, tumour 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 tumour samples contained at least 70% tumour 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 harboured *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 tumours 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 tumour 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 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 tumour types has shown that also in *PIK3CA* wild type, *KRAS* mutated colorectal cancers, a combination of PI3K/MEK inhibitors results in greater tumour 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 tumours 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 tumour 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 assumed to be a driver mutation in the majority of these tumours. 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 tumour 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 tumours and tumour 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 tumourigenesis. *HRAS* is a proto-oncogene, that, when mutated, directs cell growth and division (28). Since *TP53* is a tumour 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 tumours are simply more susceptible to additional mutations because of a lack of cell cycle control. The reported worse prognosis in tumours with multiple mutations could therefore also be a reflection of further progression of the tumour.

The fact that additional mutations in *CDKN2A* and *HRAS* occur this 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 tumour growth in VSCCs, or that this aggressiveness is caused by the additional mutations that occur later on in tumour 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 multicentre 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.

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Supplementary tables

Supplemental Table 1 Overview of detected mutations.

Sample	Patient age	FIGO Stage	HPV	Gene	AA mutation	CDS mutation
1	51	1	negative	failed for all assays		
2	77	4	negative	TP53	p.R273C	c.817C>T
3	74	4	negative	HRAS	p.G13S	c.37G>A
3	74	4	negative	TP53	p.R273H	c.818G>A
4	85	2	negative	TP53	p.Q136X	c.406C>T
5	62	2	negative	TP53	p.C135F	c.404G>T
6	78	4	negative	HRAS	p.G13D	c.38>G>A
7	54	1	negative	TP53	p.D259H	c.775G>C
8	82	2	negative	TP53	p.R248Q	c.743G>A
9	82	3	negative	TP53	p.D148N	c.442G>A
9	82	3	negative	TP53	p.R282W	c.844C>T
10	73	2	negative	TP53	p.G245S	c.733G>A
10	73	2	negative	TP53	p.V216M	c.646G>A
11	78	4	negative	TP53	p.R248Q	c.743G>A
11	78	4	negative	TP53	p.V172I	c.514G>A
12	60	2	positive	no mutation detected		
13	76	3	negative	HRAS	p.G12S	c.34G>A
13	76	3	negative	PIK3CA	p.E545K	c.1633G>A
13	76	3	negative	TP53	p.R273C	c.817C>T
14	69	2	positive	TP53	p.R175C	c.523C>T
15	73	1	negative	no mutation detected		
16	48	3	negative	HRAS	p.Q61R	c.182A>G
16	48	3	negative	PIK3CA	p.E545K	c.1633G>A
16	48	3	negative	TP53	p.P250L	c.749C>T
17	75	3	negative	TP53	p.C135F	c.404G>T
18	37	3	negative	no mutation detected		
19	54	3	negative	no mutation detected		
20	84	2	negative	PIK3CA	p.H1047L	c.3140A>T
20	84	2	negative	TP53	p.P152L	c.455C>T
21	55	1	negative	no mutation detected		
22	54	4	negative	HRAS	p.G12S	c.34G>A
22	54	4	negative	TP53	p.G245S	c.733G>A
22	54	4	negative	TP53	p.V173G	c.518T>G
23	38	1	negative	no mutation detected		
24	85	3	positive	no mutation detected		
25	53	2	negative	no mutation detected		
26	82	1	negative	no mutation detected		
27	66	1	negative	no mutation detected		
28	82	2	negative	KRAS	p.G12V	c.35G>T
28	82	2	negative	TP53	p.R248W	c.742C>T
29	54	3	negative	no mutation detected		
30	79	3	negative	CDKN2A	p.R80*	c.238C>T
31	83	3	negative	no mutation detected		
32	54	1	positive	no mutation detected		
33	80	3	negative	TP53	p.R273C	c.817C>T
34	77	2	negative	CDKN2A	p.R80*	c.238C>T
34	77	2	negative	TP53	p.P152fs ins A	c.454_455insA

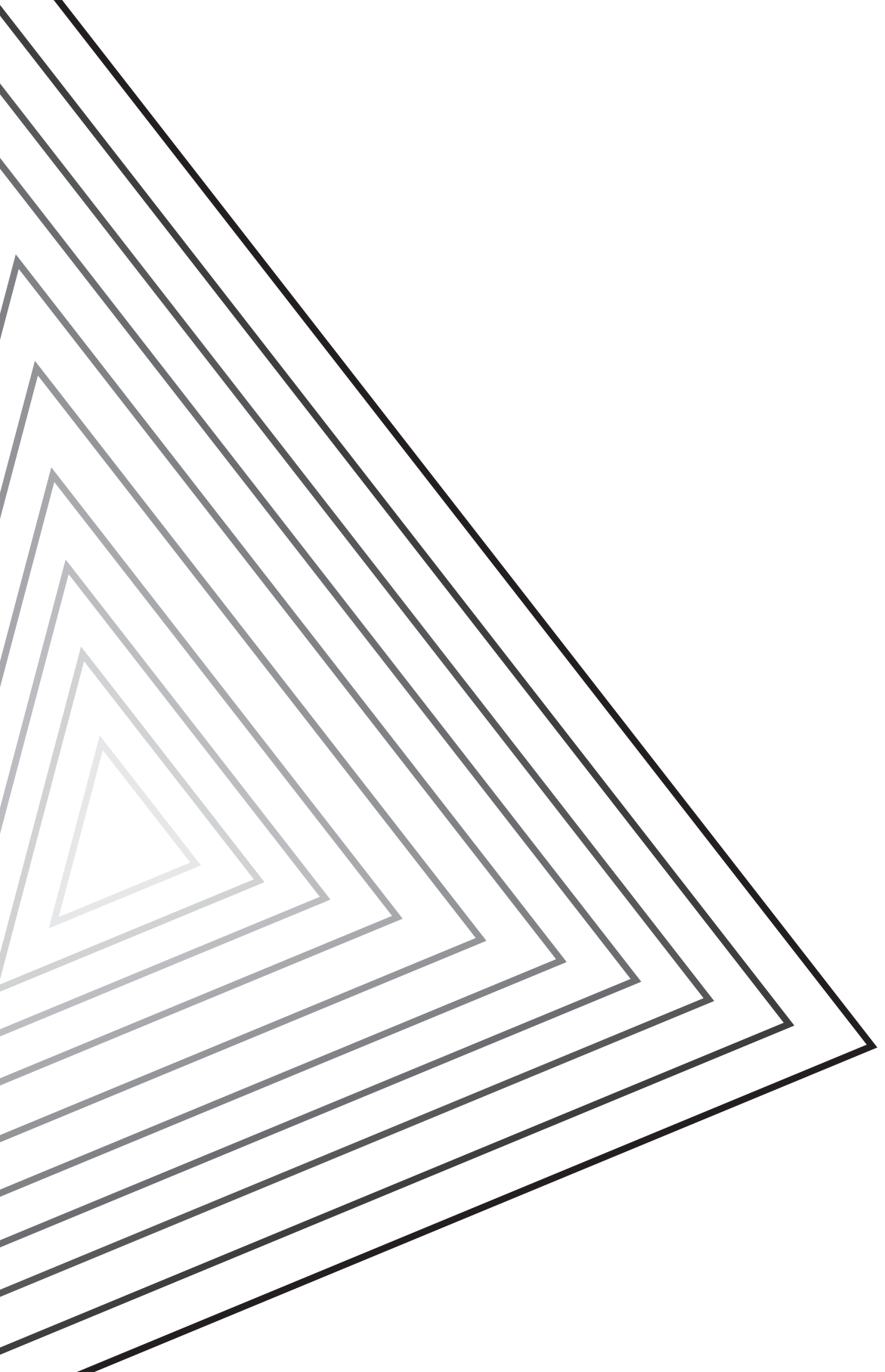
Supplemental Table 1 Continued

Sample	Patient age	FIGO Stage	HPV	Gene	AA mutation	CDS mutation
35	52	1	negative	TP53	p.D186fs ins TAGC	c.552-555dupTAGC
36	72	3	negative	CDKN2A	p.R80*	c.238C>T
36	72	3	negative	HRAS	p.G13R	c.37G>C
36	72	3	negative	TP53	p.P151S	c.451C>T
37	44	1	negative	PIK3CA	p.E545K	c.1633G>A
38	91	2	negative	CDKN2A	p.D108H	c.322G>C
39	82		negative	CDKN2A	p.R58*	c.172C>T
39	82	4	negative	TP53	p.E294X	c.880G>T
40	60	1	negative	PPP2R1A	p.R258H	c.773G>A
40	60	1	negative	TP53	p.R156C	c.466C>T
41	89	4	negative	no mutation detected		
42	69	1	negative	PPP2R1A	p.R258H	c.773G>A
42	69	1	negative	TP53	p.P152L	c.455C>T
42	69	1	negative	TP53	p.R248Q	c.743G>A
43	82	3	negative	TP53	p.T150fs*16	c.448_460del13
44	69	2	negative	TP53	p.R249S	c.747G>T
45	77	2	negative	TP53	p.C176F	c.527G>T
46	84	2	negative	no mutation detected		
47	81	1	positive	no mutation detected		
48	64	1	positive	no mutation detected		
49	59	1	negative	HRAS	p.G12S	c.34G>A
50	51	2	negative	no mutation detected		
51	94	3	negative	no mutation detected		
52	83	2	positive	no mutation detected		
53	80	2	negative	TP53	p.C176X	c.528C>A
54	83	4	negative	CDKN2A	p.R58*	c.172C>T
54	83	4	negative	TP53	p.N267fs*3	c.801_802del2
55	75	3	negative	CDKN2A	p.R80*	c.238C>T
55	75	3	negative	TP53	p.R273H	c.818G>A
56	72	2	negative	no mutation detected		
57	83	4	negative	CDKN2A	p.R80*	c.238C>T
57	83	4	negative	TP53	p.R273C	c.817C>T
58	72	1	positive	TP53	p.R248Q	c.743G>A
59	68	1	negative	TP53	p.G245S	c.733G>A
60	82	4	negative	TP53	p.C135X	c.405C>A
61	80	3	negative	CDKN2A	p.W110*	c.330G>A
61	80	3	negative	HRAS	p.G12S	c.34G>A
61	80	3	negative	TP53	p.V203L	c.607G>T
62	51	2	positive	no mutation detected		
63	88	2	negative	HRAS	p.G12S	c.34G>A
63	88	2	negative	PIK3CA	p.E545K	c.1633G>A
64	85	2	negative	TP53	p.R175H	c.524G>A
65	62	1	negative	TP53	p.E287D	c.861G>C
65	62	1	negative	TP53	p.E294X	c.880G>T
66	86	3	negative	CDKN2A	p.R80*	c.238C>T
66	86	3	negative	TP53	p.R248W	c.742C>T
67	79	3	negative	TP53	p.R248Q	c.743G>A
68	81	2	negative	TP53	p.R306X	c.916C>T
69	91	3	negative	TP53	p.V173L	c.517G>T
70	76	2	negative	no mutation detected		

Supplemental Table 1 Continued

Sample	Patient age	FIGO Stage	HPV	Gene	AA mutation	CDS mutation
71	72	2	negative	TP53	p.C242S	c.725G>C
72	58	2	negative	PIK3CA	p.H1047Y	c.3139C>T
72	58	2	negative	PPP2R1A	p.R258H	c.773G>A
73	78	2	negative	TP53	p.R248W	c.742C>T
74	58	3	negative	TP53	p.V157F	c.469G>T
75	53	1	positive	no mutation detected		
76	59	1	negative	TP53	p.E286K	c.856G>A
77	49	1	positive	no mutation detected		
78	64	2	negative	no mutation detected		
79	78	3	negative	TP53	p.G266E	c.797G>A
80	76	3	negative	CDKN2A	p.R58*	c.172C>T
80	76	3	negative	PTEN	p.R234W	c.700C>T
80	76	3	negative	TP53	p.R273C	c.817C>T
81	81	1	positive	no mutation detected		
82	73	3	negative	TP53	p.D281E	c.843C>G
82	73	3	negative	TP53	p.N239S	c.716A>G
83	79	2	negative	TP53	p.R175H	c.524G>A
84	77	1	negative	TP53	p.Y205C	c.614A>G
85	45	1	positive	no mutation detected		
86	78	2	negative	no mutation detected		
87	38	3	negative	HRAS	p.G12D	c.35G>A
88	74	1	negative	no mutation detected		
89	48	1	positive	no mutation detected		
90	59	4	negative	no mutation detected		
91	53	1	positive	no mutation detected		
92	50	1	positive	no mutation detected		
93	73	3	negative	TP53	p.P190L	c.569C>T
94	92	3	negative	CDKN2A	p.R80*	c.238C>T
94	92	3	negative	TP53	p.R273C	c.817C>T
95	85	2	negative	TP53	p.E286K	c.856G>A
96	61	4	negative	no mutation detected		
97	78	1	negative	CDKN2A	p.R58*	c.172C>T
97	78	1	negative	PIK3CA	p.E545K	c.1633G>A
97	78	1	negative	TP53	p.E285K	c.853G>A
98	83	3	negative	no mutation detected		
99	47	2	negative	TP53	p.S241Y	c.722C>A
100	75	2	positive	TP53	p.S241F	c.722C>T
101	77	2	positive	no mutation detected		
102	80	2	negative	TP53	p.A138V	c.413C>T
103	63	2	negative	TP53	p.H178Q	c.534C>A
103	63	2	negative	TP53	p.S183X	c.548C>A
104	80	3	negative	CDKN2A	p.R80*	c.238C>T
105	85	3	negative	TP53	p.R175H	c.524G>A
106	74	3	negative	no mutation detected		
107	39	2	negative	no mutation detected		
108	55	1	negative	TP53	p.C176X	c.528C>A

Lists all detected mutations, age and FIGO stage per patient. FIGO = International Federation of Gynecology and Obstetrics, AA = amino acid, CDS = coding DNA sequence



Chapter 4

Genetic and epigenetic changes in vulvar squamous cell carcinoma and its precursor lesions: A review of the current literature

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Gynecologic Oncology, Volume(s) 136, 14-Nov-2014, Pages 143-157

Abstract

Vulvar cancer is a relatively rare gynaecologic malignancy with an annual incidence in developed countries of approximately 2 per 100,000 women. Vulvar squamous cell carcinoma (VSCC) has two etiological pathways: a high risk human papillomavirus (HPV)-dependent route, which has usual vulvar intraepithelial neoplasia (uVIN) as a precursor lesion, and an HPV-independent route, which is associated with differentiated VIN (dVIN), lichen sclerosus, and genetic alterations, such as *TP53* mutations. Research on the molecular etiology of vulvar cancer has increased in past years, not only regarding genetic alterations, but also epigenetic changes. In genetic alterations, a mutation irreversibly changes the nucleotide sequence of the DNA, or the number of copies of chromosomes per cell is altered. In epigenetics, the nucleotide sequence remains the same but genes can be 'switched' on or off by, for example, DNA methylation or histone modification. We searched the current literature on genetic and epigenetic alterations in VSCC and its precursor lesions. Many studies have reported a higher incidence of somatic mutations in HPV-negative tumours compared to HPV-positive tumours, with *TP53* mutations being the most frequent. These somatic mutations seem to occur more often with increasing grades of dysplasia. Allelic imbalances or loss of heterozygosity are more frequently found in higher stages of dysplasia and in invasive carcinomas, but it is not exclusive to HPV-negative tumours. A limited number of studies are available on epigenetic changes in vulvar lesions, with hypermethylation of *CDKN2A* being the most frequently investigated change. For most genes, hypermethylation occurs more frequently in VSCC than in precursor lesions. As most studies have focused on HPV infection and *TP53* mutations, we suggest that more research should be performed using whole genome or next generation sequencing to determine the true landscape of genetic and epigenetic alterations in VSCC.

Introduction

Vulvar cancer is a rare malignant disease accounting for less than 5% of gynaecological malignancies (1-3). The majority of these tumours are vulvar squamous cell carcinoma (VSCC). The annual incidence of VSCC in developed countries is two to three per 100,000 women and increases with age, with a peak incidence between 60 and 70 years of age (1;4;5).

The pathogenesis of VSCC can be subdivided into two different pathways: human papillomavirus (HPV)-dependent and HPV-independent (1-7). The HPV-dependent pathway accounts for 20-40% of VSCCs and has usual vulvar intraepithelial neoplasia (uVIN) as a precursor lesion (3;4;8). This pathway is more common in younger women and is associated with smoking, a higher number of sexual partners, and a compromised immune status (1;3;9). The incidence of VIN, especially the usual type, has increased in the last couple of years, even doubling in some countries (1;4-6). The risk of the progression of a uVIN lesion towards VSCC seems low, occurring in 9-16% of patients who do not receive treatment and in approximately 3% of patients who have been treated (1;6). However, some studies have reported a higher risk of progression (10;11).

The non-HPV pathway is associated with mutations in *TP53* and mainly occurs in older women (1-3;6;7). This pathway is associated with lichen sclerosus (LS), a chronic dermatosis associated with autoimmune diseases. Approximately 3-5% of women with LS progress towards VSCC (9;12). Differentiated VIN (dVIN) is considered to be a precursor lesion of HPV-independent VSCC, with a higher malignant potential than uVIN (1;6). dVIN can be difficult to diagnose for both clinicians and pathologists because of its subtle clinical and histological appearance (13). HPV-independent VSCC is associated with a worse prognosis than HPV-associated VSCC (3;9). However, its carcinogenesis has not been fully clarified.

When diagnosed at an early stage, VSCC has a good prognosis, especially for patients without inguinofemoral lymph node metastasis at first presentation (14). Unfortunately, approximately one-third (15) of patients suffer from recurrent disease. In the latter group of patients, therapeutic options are limited due to severe morbidity associated with repeated treatment of local recurrences. Recurrent disease in inguinal lymph nodes has a very poor prognosis and is almost always fatal (16;17). Information on genetic and epigenetic changes that play a role in the carcinogenesis of vulvar cancer may provide valuable insight into its etiology. Studies of many different types of cancer have shown that genetic and epigenetic alteration status can help predict prognosis and guide targeted therapy (18-23). For example, vemurafenib, a BRAF inhibitor, has shown clinical efficacy as targeted therapy for melanomas that harbour mutations in *BRAF* (24). In HPV-negative VSCC, mutations are often found in the tumour suppressor gene *TP53* (1;8;9;25;26). *TP53* mutations are thought to be an early event in the

development of VSCC because they are also found in dVIN and LS lesions (1;6-8;26). Other mutations have been described in VSCC and its precursor lesions, including mutations in the tumour suppressor genes *PTEN* and *CDKN2A* (27;28). Other types of genetic alterations are allelic imbalances or copy number alterations, in which the number of copies of chromosomes per cell is altered. In addition to genetic mutations, epigenetic changes may also play a role in the development of VSCC. Epigenetic changes are defined as heritable changes in gene expression without changes in the DNA sequence. The best known epigenetic change is hypermethylation of CpG islands in the promoter regions of tumour suppressor genes, causing inactivation of the gene (19;23;29-32). In vulvar cancer, hypermethylation of the promoters of *RASSF2A*, *MGMT,* and *TSP1* has been described (30). Here, we review the current literature and summarize the current understanding of the role of genetic and epigenetic changes in VSCC and its precursor lesions.

Materials and methods

Relevant studies on genetic alterations (somatic mutations, allelic imbalances, loss of heterozygosity, copy number changes, and microsatellite instability) and epigenetic changes (hypomethylation and hypermethylation, microsatellite instability, and chromatin, histone, and posttranscriptional modifications) were identified from an extensive search on PubMed, Embase, Web of Science, Cochrane, and ScienceDirect. After consulting a medical librarian, a combination of Medical Subject Headings (MeSH) and free text words were formulated. Our search included the terms vulvar neoplasm, vulvar carcinoma, vulvar intraepithelial neoplasia, lichen sclerosus et atrophicus, mutation, microsatellite instability, genetic, epigenetic, hypermethylation, chromatin, histone, and posttranscriptional modifications. Research published until 31 July 2014 that studied somatic mutations and epigenetic changes in VSCC, VIN, and/or LS were included in this review. Exclusion criteria were languages other than English, Dutch, German, French, or Italian, meeting abstracts, or if the researchers only performed immunohistochemistry to evaluate protein function. Two researchers (MDT and LN) independently assessed all articles based on the title, abstract, or full article. Articles for which there was disagreement regarding inclusion or exclusion were discussed and a consensus reached. The electronic search was complemented by a manual search of bibliographies from relevant articles in order to identify additional relevant studies not encountered in the electronic search. The articles that met all inclusion criteria are described in this review.

Results

The electronic search identified 198 articles on genetic alterations in VSCC, VIN, and LS. The manual search yielded another 17 articles. 59 of these articles met the inclusion criteria and were included in this review (Tables 1 and 3). For epigenetic changes in VSCC, VIN, and LS, we found 49 articles, nine of which are included in this review (Table 4). Four articles reported on both genetic and epigenetic changes and are found in both Table 1 and Table 4 (28;33-35). A flowchart illustrating the inclusion and exclusion of articles is shown in Figure 1.

Figure 1 *Inclusion and exclusion of articles.*

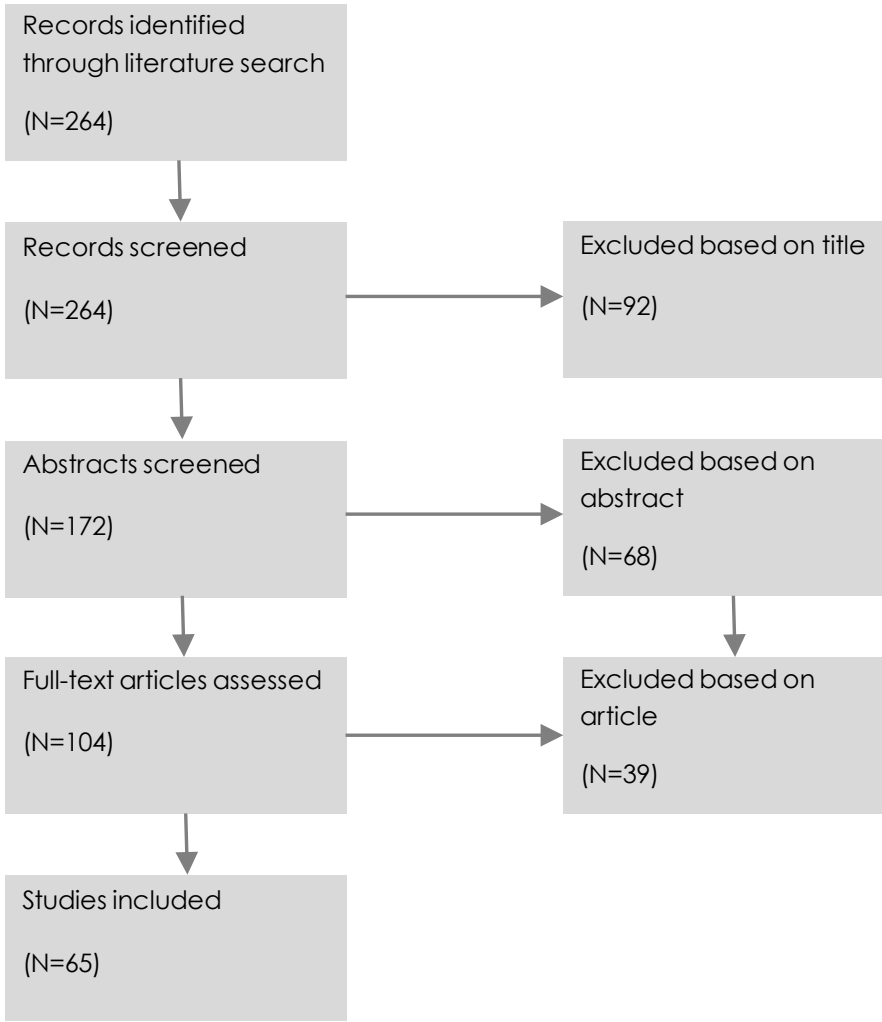

Table 1 *Studies on mutations in vulvar cancer and its precursors.*

Author	Year	No. of patients	Diagnosis	HPV-status	Gene	Mutation %	Technique used	Remarks
Pilotti	1993	5	verrucous VC	-	*TP53*	0%	SSCP exon 5-9 + confirmation sequencing	
Kurvinen	1994	1	CIS	+	*TP53*	0%	SSCP exon 5-9 + confirmation sequencing	
		1	VIN	+	*TP53*	0%		
		2	VSCC	-	*TP53*	0%		
		7	VSCC	+	*TP53*	0%		
Lee	1994	9	VSCC	-	*TP53*	44%	SSCP exon 5-8 and part of exon 4	
		12	VSCC	+	*TP53*	8%		
Milde-Langosch	1995	12	VIN	50%*	*TP53*	33%	PCR-TGGE	* not described in association to mutations
Pilotti	1995	7	VIN*	+	*TP53*	0%	SSCP exon 5-9	*some adjacent to reported VSCC
		12	VSCC	-	*TP53*	33%		
		4	VSCC	+	*TP53*	50%		
Kim	1996	11	VSCC	-	*TP53*	36% (25% keratinising, 100% Pagets)	SSCP exon 5-8	* 11 (8 keratinising, 1 basaloid, 2 Pagets) 7 (3 keratinising, 2 basaloid, 1 Pagets, 1 warty)
		7	VSCC	+	*TP53*	0%		
Sliutz	1997	38	VSCC	not tested	*TP53*	32%	PCR-TGGE	
Wong	1997	6	VSCC	not tested	*CDKN2A and CDKN2B*	0%	SSCP *CDKN2A* exon 1-3 and *CDKN2B* exon 1-2	
Flowers	1999	10*	VIN	-	*TP53*	10%		* multiple samples from same patient
		11*	VIN	+	*TP53*	9%		
		15	VSCC	-	*TP53*	29% KSC, 0% basaloid		
		15	VSCC	+	*TP53*	33% KSC, 8% basaloid		
Ngan	1999	25	VSCC	-	*TP53*	20%	SSCP exon 5-8 + confirmation sequencing	

Table 1 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene	Mutation %	Technique used	Remarks
		23	VSCC	+	*TP53*	22%		
Brooks	2000	23	VSCC	-	*TP53*	74%	SSCP exon 4-9	codon 72P/R same cohort as Marin 2000 and O'Nion 2001
		13	VSCC	+	*TP53*	31%		
Holway	2000	2*	VIN	not tested	*PTEN*	100%	SSCP exon 5-8	* same patients as VSCC
		10	VSCC	not tested	*PTEN*	60%		1 patient had PTEN mutation in VIN but not in adjacent VSCC. In 3 patients different mutations were found in VIN and VSCC
Marin	2000	36	VSCC	not tested	*TP53*	58%	SSCP exon 4-9 + confirmation sequencing	
		10	LS	-	*TP53*	70%		
		29 (3 basaloid, 26 squamous)	VC	-	*TP53*	55%		
		11 (3 basaloid, 8 squamous)	VC	+	*TP53*	45%		
Wada	2000	1	VIN	+	*TP53* + *KRAS*	0% *TP53*, 0% *KRAS*	SSCP *TP53* exon 5-8, KRAS exon 1	
O'Nions	2001	23	VSCC	-	*TP53* + *CDKN2A*	74% *TP53*, 13% *CDKN2A*	SSCP CDKN2A exon 1a + 2, TP53 exon 7-9	
		13	VSCC	+	*TP53* + *CDKN2A*	31% *TP53*, 0% *CDKN2A*		
Gasco	2002	23	VSCC	-	*CDKN2A* + *Stratifin* +*TP53*	13% *CDKN2A*, 0% *Stratifin*, 73,9 % *TP53*		
		20	VIN	-	*CDKN2A* + *Stratifin* +*TP53*	0% *CDKN2A*, 0% *Stratifin*, 0% *TP53*		*CDKN2A* and *stratifin* were tested on 11 patients
		12	VIN	+	*CDKN2A* + *Stratifin* + *TP53*	0% *CDKN2A*, 0% *Stratifin*, 0% *TP53*		*CDKN2A* and *stratifin* were tested on 11 patients
		13	VSCC	+	*CDKN2A* + *Stratifin* +*TP53*	0% *CDKN2A*, 0% *Stratifin*, 30,8% *TP53*		

Table 1 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene	Mutation %	Technique used	Remarks
Rampone	2002	8	LS	not tested	*TP53*	63%	Sanger sequencing exon 5-9	
		10	LSC	not tested	*TP53*	0%		
Reddy	2002	32	VIN	not tested	*CHK2*	0% *CHK2*		
		40	VSCC	not tested	*CHK2* + *TP53*	5 % *CHK2*, 100% *TP53* *	SSCP *CHK2* exon 1a, 1b, 2-14, TP53	* only tested in *CHK2* mutated samples
Vanin	2002	62*	LS	-	*TP53*	5%	Sanger sequencing exon 5-8	* 25 with VSCC, 37 without VSCC
		29	VSCC	-	*TP53*	28%		
Rolfe	2003	12	LS	not tested	*TP53*	58%	Sanger sequencing exon 5-8	
		27	VSCC	not tested	*TP53*	81%		
Almeida	2004	2	undifferentiated VIN	-	*TP53*	50%	SCCP exon 5-8	
		6	undifferentiated VIN	+	*TP53*	17%		
Chulvis do Val	2004	13	undifferentiated VIN	64%*	*TP53*	38%	SSCP exon 5-8	* not described in association to mutations
Olawaiye	2007	2	VSCC	not tested	*EGFR*	0%	Sanger sequencing exon 18-24	
Osakabe	2007	16	VSCC	-	*TP53*	63%	SCCP exon 5-8	
		5	VSCC	+	*TP53*	20%		
		7	Bowenoid early invasion and 1 invasive SCC	+	*TP53*	0%		
Soufir	2007	21	LS	not tested (not for all)	*CDKN2A* + *TP53*	0% *CDKN2A*, 0% *TP53*	SSCP *CDKN2A* exon 1a, 1β and 2, *TP53* exon 4-9	
		2	VIN	not tested (not for all)	*CDKN2A* + *TP53*	0% *CDKN2A*, 0% *TP53*		
		5	VSCC	not tested (not for all)	*CDKN2A* + *TP53*	20% *CDKN2A*, 60% *TP53*		

Table 1 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene	Mutation %	Technique used	Remarks
Tapp	2007	224	LS	not tested	*TP53 + KRAS (2+1 hotspot codons only)*	0% had a single mutant population that exceeded 20 per 10^6	PCR/RE/LCR	reports SBS single base instability (not somatic mutations, but 1 in a million errors) and only looked at 2 hotspots in *TP53* (codon 248 and 273) and 1 in *KRAS* (codon 12)
Aulman	2008	12	VIN (7 uVIN, 5 dVIN)	-	*TP53*	17%	SSCP exon 4-10	
		20	uVIN	+	*TP53*	0%		
		24	VSCC	-	*TP53*	17%		
		4	VSCC	+	*TP53*	0%		
Growdon	2008	19	VSCC	-	*EGFR*	0%	Sanger sequencing exon 18-21	
		22	VSCC	+	*EGFR*	0%		
		5*	CIS	not tested	*PTEN*	60%		
Pinto	2010	11	VIN	-	*TP53*	60%	Sanger sequencing	
		5	VSCC	-	*TP53*	80%		
Choschzick	2011	21	VSCC	-	*TP53*	77%	Sanger sequencing exon 5-8	
		18	VSCC	+	*TP53*	24%		
Janku	2011	2	VSCC	not tested	*PIK3CA*	0%	Sanger sequencing c532-554 of exon 9 and c1011-1062 of exon 20	
Horowitz	2012	17	VSCC	not tested	*EGFR*	0%	Sanger sequencing	
Gambichler	2013	10	LS	not tested	*TP53, NRAS, KRAS, IDH1, IDH2, TET2*	0%	Sanger sequencing *IDH1* exon 4, *IDH2* exon 4, *TET2* exon 3 + 11, *TP53* exon 4,6,7, *KRAS* codon 12, *HRAS* exon 3, *NRAS* exon 2-3	
		5	CIS	-	*EGFR*	0%		
		5	CIS	+	*EGFR*	0%		

Table 1 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene	Mutation %	Technique used	Remarks
Trietsch	2014	89	VSCC*	-	*BRAF, CDKN2A, CTNNB1, FBXW7, FGFR2, FGFR3, FOXL2, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A, PTEN, and TP53*	0% *BRAF*, 16% *CDKN2A*, 0% *CTNNB1*, 0% *FBXW7*, 0% *FGFR2*, 0% *FGFR3*, 0% *FOXL2*, 11% *HRAS*, 1% *KRAS*, 0% *NRAS*, 8% *PIK3CA*, 3% *PPP2R1A*, 1% *PTEN*, 62% *TP53*	Hot spot mass spectrometry, Sanger sequencing *TP53 exon 5-9*	*Partial overlap in VSCC patients reported in a recent article by Spaans et al. (1)
		18	VSCC*	+	*BRAF, CDKN2A, CTNNB1, FBXW7, FGFR2, FGFR3, FOXL2, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A, PTEN, and TP53*	0% *BRAF*, 0% *CDKN2A*, 0% *CTNNB1*, 0% *FBXW7*, 0% *FGFR2*, 0% *FGFR3*, 0% *FOXL2*, 0% *HRAS*, 0% *KRAS*, 0% *NRAS*, 0% *PIK3CA*, 0% *PPP2R1A* 0% *PTEN*, 17% *TP53*		

HPV= human papillomavirus, N= number, LS= lichen sclerosus, LSC= lichen sclerosus chronicans, VSCC= vulvar squamous cell carcinoma, VIN= vulvar intraepithelial neoplasia, uVIN= usual vulvar intraepithelial neoplasia, dVIN= differentiated vulvar intraepithelial neoplasia, CIS= carcinoma in situ, SCCP= single strand confirmation polymorphism, PCR= polymerase chain reaction, TGGE= temperature gradient gel electrophoresis, KSC= keratinizing squamous carcinoma, LCR= ligand chain reaction, RE= restriction endonuclease
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for mutations

Table 2 *Overall mutation frequencies*

	LS				VIN						VSCC					
	HPV neg		HPV unknown		HPV pos		HPV neg		HPV unknown		HPV pos		HPV neg		HPV unknown	
TP53	10/72	14%	12/285	4%	2/66	3%	10/47	21%	11/29	38%	28/171	16%	109/361	30%	28/108	26%
PTEN									2/2	100%	0/18	0%	1/89	1%	6/10	60%
EGFR											0/22	0%	0/19	0%	0/19	0%
BRAF											0/18	0%	0/89	0%		
HRAS											0/18	0%	10/89	11%		
KRAS			0/10	0%							0/18	0%	1/89	1%		
NRAS			0/10	0%							0/18	0%	0/89	0%		
CDKN2A			0/21	0%	0/4	0%	0/2	0%	0/2	0%	0/44	0%	20/135	15%	1/11	9%
CTNNB1											0/18	0%	0/89	0%		
PPP2R1A											0/18	0%	3/89	3%		
FBXW7											0/18	0%	0/89	0%		
PIK3CA											0/18	0%	7/89	8%	0/2	0%
IDH1			0/10	0%												
IDH2			0/10	0%												
TET2			0/10	0%												
CHK2									0/32	0%					2/40	5%
FGFR2											0/18	0%	0/89	0%		
FGFR3											0/18	0%	0/89	0%		
FOXL2											0/18	0%	0/89	0%		
Stratifin					0/4	0%	0/2	0%			0/13	0%	0/23	0%		

LS= lichen sclerosus, VIN= vulvar intraepithelial hyperplasia, VSCC= vulvar squamous cell carcinoma, HPV= human papillomavirus
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for mutations

Table 3 *Studies on allelic imbalances in vulvar cancer and its precursors.*

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
Wong	1997	6	VSCC	not tested	*CDKN2A* and *CDKN2B*	50% *CDKN2A*, 50% *CDKN2B*	loss	LOH	
Lin	1998	2	VIN	-		0% 1.2, 0% 2.3, 50% 2.4, 0% 3.1, 0% 3.4, 0% 4.1, 50% 5.2, 50% 5.3, 0% 8.2, 0% 21.1	loss	LOH	
		2	VIN	+		0% 1.2, 50% 2.3, 50% 2.4, 0%3.1, 50% 3.4, 0% 4.1, 0% 5.2, 0% 5.3, 50% 8.2, 0% 21.1	loss		
		2	VSCC	-		0% 1.2, 100% 2.3, 100% 2.4, 50% 3.1, 50% 3.4, 50% 4.1, 100% 5.2, 50% 5.3, 50% 8.2, 50% 21.1	loss		

Table 3 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
		2	VSCC	+		50% 1.2, 0% 2.3, 100% 2.4, 0% 3.1, 0% 3.4, 0% 4.1, 0% 5.2, 100% 5.3, 50% 8.2, 0% 21.1	loss		
Flowers	1999	10*	VIN	-	3p chromosomal regions (3p 12, 3pl 4.2, 3pl 4.3-21.1, 3p2l.3, 3p22-24, 3p24.3, 3p25), 13q14 (*RB*) and 17pl3.1 (*TP53*) loci	54% 3p, 14% 13q (*RB*), 9% 17p (*TP53*)	loss	LOH	* multiple samples from same patients
		10*	VIN	+	3p chromosomal regions (3p 12, 3pl 4.2, 3pl 4.3-21.1, 3p2l.3, 3p22-24, 3p24.3, 3p25), 13q14 (*RB*) and 17pl3.1 (*TP53*) loci	16% 3p, 6% 13q (*RB*), 0% 17p (*TP53*)	loss		
		15	VSCC	-	3p chromosomal regions (3p 12, 3pl 4.2, 3pl 4.3-21.1, 3p2l.3, 3p22-24, 3p24.3, 3p25), 13q14 (*RB*) and 17pl3.1 (*TP53*) loci	93% 3p, 27% 13q (*RB*), 62% 17p (*TP53*)	loss		
		15	VSCC	+	3p chromosomal regions (3p 12, 3pl 4.2, 3pl 4.3-21.1, 3p2l.3, 3p22-24, 3p24.3, 3p25), 13q14 (*RB*) and 17pl3.1 (*TP53*) loci	67% 3p, 31% 13q (*RB*), 15% 17p (*TP53*)	loss		
Scheistroen	1999	167	VSCC	not tested		77 % diploid, 23% aneuploid		FACS	

Table 3 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
Pinto	1999	8	VSCC	-		Overall 36% LOH. Most frequent: 83% 5q, 100% 10p, 29% 1p, 25% 2q, 50% 3p, 63% 8p, 63% 8q, 60% 10q, 50% 11q, 29% 15q, 80% 17p, 50% 21q, 60% 22q.	loss	LOH	
		8	VSCC	+		Overall 30% LOH. Most frequent: 13% 5q, 17% 10q, 33% 1p, 0% 2q, 50% 3p, 13% 5q, 33% 8p, 50% 8q, 17% 10p, 25% 11q, 43% 15q, 43% 17p, 67% 21q, 20% 22q	loss		
Pinto	2000	16	VIN (5 uVIN, 11 dVIN)	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	15%*	both	LOH	*scoring informative (heterozygous) loci
		14	VIN (10 uVIN, 4 dVIN)	+	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	25%*	both		*scoring informative (heterozygous) loci

Table 3 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
		17	LS	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	10%*	both		*scoring informative (heterozygous) loci
Brooks	2000	23	VSCC	-	TP53	61%	loss	LOH	codon 72P/R same cohort as Marin 2000 and O'Nion 2001
		13	VSCC	+	TP53	54%	loss		
Carlson	2000	12	LS	not tested	chr 17	chr 17 aneusomy:100%. DNA index aneuploidy: 58%		FISH	
		3	VIN	not tested	chr 17	chr 17 aneusomy: 100% DNA index aneuploidy: 67%			
		14*	VSCC	not tested	chr 17	chr 17 aneusomy: 93%. DNA index aneuploidy: 86%			* 10 SCC, 4 SCCIS
Marin	2000	36	VSCC	not tested	TP53	54%	loss	LOH	
Wada	2000	1	VIN	+	3p14.2, 3p, 9p21, 9p23, 13q22, 17p12	0%	loss	LOH	
Jee	2001	10	VSCC	not tested		DNA copy number changes in 80%. Loss: 50% 4p13-pter, 40% 3p, 10% 5q14-q23, 10% 6q11-q16, 10% 11q21-qter, 10% 13q14-q32. Gain: 40% 3q, 30% 8q, 10% 9p, 10% 14, 10% 17, 10% 20q	both	CGH	

Table 3 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
Rosenthal	2001	13	VSCC	-		LOH of 48% 17p, 40% 9p, 48% 3p, 44% 4q, 43% 5p, 44% 11p	loss		
		54	VSCC	+		LOH of 48% 17p, 40% 9p, 48% 3p, 44% 4q, 43% 5p, 44% 11p	loss		
Allen	2002	8	VSCC	-		Most common: 75% 8q gain, 0% 3q gain, 13% 3p loss, 50% 11q loss	both	CGH	
		10	VSCC	+		Most common 20% 8q gain, 50% 3q gain, 40% 3p loss, 40% 11q loss	both		
Reddy	2002	32	VIN	not tested	CHK2	0%*			
		40	VSCC	not tested	CHK2	2%*	loss	direct sequencing of RT-PCR product	* only tested in *CHK2* mutated samples
Vanin	2002	62*	LS	-	TP53	0%	loss	LOH	* 25 with VSCC, 37 without VSCC
		29	VSCC	-	TP53	74%	loss		
Bryndorf	2004	4	condyloma	-		0 chromosomal aberations	both	hrCGH and FACS	

Table 3 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
		2	VIN	-		100% diploid. Most common gain of: 0% chr 1, 0% 3q, 0% 20q, 0% 20p, 0% 3q, 0% 8q. Loss of 0% 3p, 0% 8p	both		
		9	VIN	+		40% diploid, 30% aneuploid, 30% tetraploid. Most common gain of: 60% chr 1, 50% 3q, 50% 20q, 40% 20p, 30% 8q. Loss of 20% 3p, 0% 8p	both		
		6	VSCC	-		25% diploid, 75% aneuploid. Most common gain of: 0% chr 1, 75% 3q, 50% 20q, 50% 20p, 100% 8q. Loss of 50% 3p, 50% 8p	both		

Table 3 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
		4	VSCC	+		50% diploid, 50% tetraploid. Most common gain of: 0% chr 1, 66% 3q, 17% 20q, 17% 20p, 33% 8q. Loss of 83% 3p, 33% 8p	both		
Huang	2005	8	VSCC	75%*		gains of 1q 13%, 3q 38%, 5p 38%, 8q 75%. Losses 3p 38%, 4p 13%, 11p 13%	both	CGH	* not described in association to genetic changes
Olawaiye	2007	2	VSCC	not tested	EGFR	0%		q rtPCR	
Osakabe	2007	16	VSCC	-		LOH of 44% 3p14.2 (*FHIT*), 38% 3p26 (*VHL*), 38% 5q31 (*APC*), 63% 9q21 (*p16*), 67% 9q22.3 (*PTECH*), 38% 10p15 (*PAHX*), 30% 13q14.3-21.1 (*Rb*), 40% 17p13 (*TP53*), 44% 18q21 (*DCC*). Fractional allelic loss 43%	loss	LOH	

Table 3 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
		5	VSCC	+		LOH of 50% 3p14.2 (*FHIT*), 100% 9q21 (*p16*), 50% 9q22.3 (*PTCH*), Fractional allelic loss 18%	loss		
Yangling	2007	10	VSCC	-	3q, 3p, 4p, 8q, 12q	Gain: 10% 3q, 70% 8q 0% 12q, Loss: 40% 3p, 50% 4p	both	CGH	
		11	VSCC	+	3q, 3p, 4p, 8q, 12q	Gain: 73% 3q, 64% 12q, 9% 8q. Loss: 46% 3p, 55% 4p	both		
Growdon	2008	19	VSCC	-	EGFR + HER2	32% *EGFR*, 0% *HER2*, 16% polysomy chr 7	gene amplification	FISH	
		22	VSCC	+	EGFR + HER2	0% *EGFR*, 0% *HER2*			
		5	CIS	-	EGFR + HER2	0% *EGFR*, 0% *HER2*			
		5	CIS	+	EGFR + HER2	0% *EGFR*, 0% *HER2*			
Aulman	2008	12	VIN (7 uVIN, 5 dVIN)	-	3q26	73%	gain	FISH	
		20	uVIN	+	3q26	50%	gain		
		24	VSCC	-	3q26	83%	gain		
		4	VSCC	+	3q26	75%	gain		

Table 3 Continued

Author	Year	No. of patients	Diagnosis	HPV-status	Gene/locus	AI %	loss or gain	Technique used	Remarks
Horowitz	2012	17	VSCC	not tested	EGFR	12%	gene amplification	FISH	
Lavorato-Rocha	2013	139	VSCC	33%*	TP53	65% normal gene / chr copy number, 19% polysomy, 9% monosomy, 6% deletion	both	FISH	* not described in association to genetic changes
Micci	2013	14	VSCC	not tested	Amongst others *FHIT*, *PTPRD*	70% aneuploid, 20% tetraploid, 10% diploid. 90% array-CGH imbalances. Loss of a region of 64% 8p23.1, 57% 8p21.3, 57% 8p12, 50% 3p14.2, 50% 3p13, 50% 8p23.3-p23.1, 50% 8p23.1-p11.23, 50% 8p11.22-p11.1, 50% 8q23.3, 50% 8q24.12-q24.22, 50% 9p23. Homozygous deletion of 29% p23 (*PTPRD*). No common amplified region.	both	arrayCGH + rtPCR + karyotyping	

HPV= human papillomavirus, N= number, LS= lichen sclerosus, LSC= lichen sclerosus chronicans, VSCC= vulvar squamous cell carcinoma, VIN= vulvar intraepithelial neoplasia, AI= allelic imbalance, LOH= loss of heterozygosity, FISH = fluorescence in situ hybridization, RT-PCR= real time polymerase chain reaction, (hr)CGH= (high resolution) comparative genomic hybridization, FACS= fluorescence-activated cell sorting, SCCIS= squamous cell carcinoma in situ Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for allelic imbalances

Somatic mutations

A total of 34 articles were included that described somatic mutations (Table 1) (8;25-28;33-61). Mutations were most often studied and detected in *TP53*, with frequencies of up to 70% for LS, 60% for VIN, and 81% for vulvar cancer. *CDKN2A* mutations were not detected in LS or VIN, but occurred in 0-60% of VSCCs. Table 2 shows the overall frequencies of mutations for all included studies. HPV-negative tumours harboured more mutations than HPV-positive tumours, and the percentage of mutated samples gradually increased with higher stages of (pre)cancerous lesions.

Allelic imbalances, loss of heterozygosity, and copy number changes

A total of 24 articles were included that reported allelic imbalances or copy number changes in vulvar cancer and its precursors (Table 3)(36;45;47-49;51;52;55;56;58;60;62-73). Allelic imbalances occurred most often on chromosomes 3, 8, 11, 13, and 17. Three studies focused on the total DNA index, and each found high percentages of aneuploidy and tetraploidy (62-64). Bryndorf was the only one to test HPV infection and found the highest percentage of aneuploidy and tetraploidy in HPV-negative VSCC. Allelic imbalances were more frequently observed in higher stages of both precancerous and cancerous lesions (63).

Microsatellite instability

We included three articles that reported on microsatellite instability (MSI) (65;74;75), a condition in which repetitive DNA sequences are susceptible to errors because the Mismatch Repair system is not functioning properly (table 4). The articles by Bujko and Lin looked at MSI in HPV-positive and negative VSCC. Bujko et al. found no MSI in the 44 patients they investigated (29 HPV-negative and 15 HPV-positive) (74). Lin reported MSI in locus 3.1 in one of two patients with HPV-positive VSCC (65). Pinto et al. focused on MSI and allelic imbalances in uVIN, dVIN and LS, and found that MSI was confined exclusively to HPV-negative dVIN and LS lesions, but did not occur in the 15 uVINs they studied (75). The data by Pinto suggest that these molecular changes are possibly early events in the HPV-independent route of vulvar carcinogenesis, and that MSI may play a role in the malignant potential of LS. However, in a small cohort of 4 patients with VSCC described by Lin et al., 2 patients with HPV-positive tumours displayed MSI as well. These data indicate that the exact role of MSI in vulvar carcinogenesis needs to be elucidated.

Table 4 *Studies on microsatellite instability (MSI) in vulvar cancer and its precursors.*

Author	Year	No. of patients	Diag-nosis	HPV-status	Locus	% MSI	Technique used
Lin	1998	2	VSCC	-	3.1	0%	PCR
		2	VSCC	+	3.1	50%	
Bujko	2012	29	VSCC	-		0%	PCR
		15	VSCC	+		0%	
Pinto	2000	5	uVIN	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	0%	PCR
		10	uVIN	+	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	0%	
		11	dVIN	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	27%	
		4	dVIN	+	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	0%	
		17	LS	-	3p, 5q, 8p, 8q, 10p, 10q, 11q, 17p, 18q, 21q, 22q	12%	

HPV= human papillomavirus, N= number, LS= lichen sclerosus, VSCC= vulvar squamous cell carcinoma, VIN= vulvar intraepithelial neoplasia, PCR= polymerase chain reaction

Epigenetic alterations

Nine articles were included that reported on epigenetic alterations in VSCC or its precursors (Table 5) (28-30;33;34;76-79). *CDKN2A* was studied most often (28-30;33;34;76;78;79). *CDKN2A* is more frequently hypermethylated in VSCC (up to 68%) and VIN (up to 72%) than in LS (up to 47%), but there is great variability in the reported frequencies. An overview of all genes tested for hypermethylation and the percentage of hypermethylation is shown in Table 6. When HPV status was not specified for all genes tested for hypermethylation, HPV status was interpreted as unknown.

Table 5 *Studies on hypermethylation in vulvar cancer and its precursors.*

Author	Year	No. of patients	Diag-nosis	HPV-status	Gene	% Hypermethylation	Technique used	Remarks
O'Nions	2001	13	VSCC	HPV 16 +	*CDKN2A*	15,4%	msPCR	
		23	VSCC	HPV 16 -	*CDKN2A*	47,8%	msPCR	
Gasco	2002	0	VIN 1	HPV 16 +	*Stratifin, CDKN2A*	0% *Stratifin*, 0% *CDKN2A*	msPCR	
		4	VIN 1	HPV 16 -	*Stratifin, CDKN2A*	0% *Stratifin*, 0% *CDKN2A*	msPCR	
		1	VIN 2	HPV 16 +	*Stratifin, CDKN2A*	0% *Stratifin*, 0% *CDKN2A*	msPCR	
		5	VIN 2	HPV 16 -	*Stratifin, CDKN2A*	40% *Stratifin*, 40% *CDKN2A*	msPCR	
		11	VIN 3	HPV 16 +	*Stratifin, CDKN2A*	45,5% *Stratifin*, 9,1% *CDKN2A*	msPCR	
		11	VIN 3	HPV 16 -	*Stratifin, CDKN2A*	72,7% *Stratifin*, 72,7% *CDKN2A*	msPCR	
		13	VSCC	HPV 16 +	*Stratifin, CDKN2A*	53,8% *Stratifin*, 15,4% *CDKN2A*	msPCR	
		23	VSCC	HPV 16 -	*Stratifin, CDKN2A*	56,5% *Stratifin*, 47,8% *CDKN2A*	msPCR	
Lerma	2002	21	LS	not tested	*CDKN2A*	42,8%	ms-PCR	
		13	9 uVIN, 4 dVIN	not tested	*CDKN2A*	69,2%	ms-PCR	
		38	VSCC	not tested	*CDKN2A*	68%	ms-PCR	
Soufir	2007	2	LS	HPV 16 +	*CDKN2A, p14*	0% *CDKN2A*, 0% *p14*	ms-PCR	
		8	LS	HPV 16 -	*CDKN2A, p14*	12,5% *CDKN2A*, 0% *p14*	ms-PCR	
		2	VIN3	HPV 16 +	*CDKN2A, p14*	0% *CDKN2A*, 0% *p14*	ms-PCR	
		2	VSCC	HPV 16 +	*CDKN2A, p14*	0% *CDKN2A*, 0% *p14*	ms-PCR	
		2	VSCC	HPV 16 -	*CDKN2A, p14*	0% *CDKN2A*, 0% *p14*	ms-PCR	
Aide	2010	15	LS	not tested	*DAPK + CDKN2A*	13% *DAPK*, 47% *CDKN2A*	ms-PCR	

Table 5 Continued

Author	Year	No. of patients	Diag-nosis	HPV-status	Gene	% Hypermethylation	Technique used	Remarks
Guerrero	2011	21	LS not associa-ted with VSCC	HPV + 25%	*RASSF1A, RASSF2A, CDKN2A, TSP-1 and MGMT*	52,4% *RASSF1A*, 0% *RASSF2A*, 19% *CDKN2A*, 52,4% *TSP-1*, 0% *MGMT*	ms-PCR	25% HPV positive, but HPV status not specified per gene investigated for hypermethylation
		12	LS associated with VSCC	not tested	*RASSF1A, RASSF2A, CDKN2A, TSP-1 and MGMT*	33,3% *RASSF1A*, 8,3% *RASSF2A*, 16,6% *CDKN2A*, 50% *TSP-1*, 41,7% *MGMT*	ms-PCR	
		1	VSCC	HPV +	*RASSF1A, RASSF2A, CDKN2A, TSP-1 and MGMT*	0% *RASSF1A*, 0% *RASSF2A*, 0% *CDKN2A*, 20% *TSP-1*, 0% *MGMT*	ms-PCR	TSP-1 hypermethylation was tested on 5 patients
		11	VSCC	HPV -	*RASSF1A, RASSF2A, CDKN2A, TSP-1 and MGMT*	45,5% *RASSF1A*, 72,7% *RASSF2A*, 54,5% *CDKN2A*, 40% *TSP-1*, 72,7% *MGMT*	ms-PCR	TSP-1 hypermethylation was tested on 25 patients
Aide	2012	23	LS	not tested	*DAPK + CDKN2A*	17% *DAPK*, 35% *CDKN2A*	ms-PCR	
Oonk	2012	20	VSCC	not tested	*CDKN2A, MGMT, TWIST1, CADM1, TERT and TFPI2*	65% *CDKN2A*, 45% *MGMT*, 35% *TWIST 1*, 55% *CADM1*, 100% *TERT*, 60% *TFPI2*	msPCR	
Guerrero	2013	21	LS	HPV + 25%	*TSLC-1*	25% *TSLC-1*	ms-PCR	25% HPV positive, but HPV status not specified per gene investigated for hypermethylation
		30	VSCC	16,7% +	*TSLC-1*	44,4% *TSLC-1*	ms-PCR	Same cohort as Guerrero 2011. Only new results are described here.

HPV= human papillomavirus, N= number, LS= lichen sclerosus, LSC= lichen sclerosus chronicans, VSCC= vulvar squamous cell carcinoma, VIN= vulvar intraepithelial neoplasia, msPCR= methylation-specific polymerase chain reaction
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for hypermethylation

Table 6 *Overall hypermethylation frequencies.*

	LS						VIN						VSCC					
	HPV pos		HPV neg		HPV unknown		HPV pos		HPV neg		HPV unknown		HPV pos		HPV neg		HPV unknown	
CDKN2A	0/2	0%	1/8	12,5%	26/92	28,3%	1/14	7,1%	10/20	50%	9/13	69,2%	4/29	13,8%	28/59	47,5%	39/58	67,2%
p14	0/2	0%	0/8	0%			0/2	0%					0/2	0%	0/2	0%		
DAPK					6/38	15,8%												
MGMT					0/33	0%											9/20	45%
TWIST1																	7/20	35%
CADM1																	11/20	55%
TERT																	20/20	100%
TFPI2																	12/20	60%
RASSF1A					15/33	45,5%												
RASSF2A					1/33	3,0%												
TSP-1					17/33	51,5%												
Stratifin							5/12	41,7%	10/20	50%			7/13	53,8%	11/23	56,5%		
TSLC-1					9/21	42,9%											11/30	44,4%

LS= lichen sclerosus, VIN= vulvar intraepithelial hyperplasia, VSCC= vulvar squamous cell carcinoma, HPV= human papillomavirus
Nb. HPV status was interpreted as unknown if it was not specified for all genes tested for hypermethylation

Discussion

A growing body of research has focused on genetic and epigenetic changes in vulvar cancer. The combined results of the currently available literature on genetic and epigenetic changes confirm the hypothesis that HPV and *TP53* mutations play almost separate, but key roles in the carcinogenesis of VSCC (Table 5). Patients infected with HPV are less likely to carry somatic mutations than patients without HPV, but allelic imbalances seem to occur in both groups. The cumulative number of genetic changes increases with increasing grade of dysplasia and cancer stage. Although only a few studies have sufficient numbers of patients to perform survival analysis related to genetic and epigenetic changes, the findings suggest that tumours harbouring a mutation, which are most often HPV-independent VSCC, have a worse prognosis than VSCC without (epi)genetic changes (36;43;50;54;58;62;73;80).

The frequencies of detected mutations vary between studies. These differences can be explained, in part, by the composition of the cohorts. The included cohorts may vary in terms of age and ethnic background or tumour stage, which is known to be related to genetic alterations. Also, differences in the techniques used and coverage of the screened exons may play a role. Detection methods have improved over the last few decades, which is reflected in an overall increase in the number of detected *TP53* mutations within HPV-negative tumour samples.

The amount of research on epigenetic changes in VSCC and its precursors is limited, but studies in other types of cancer have shown the importance of these tumour characteristics in the development of targeted therapy (81). We only found articles on hypermethylation. In our literature search we did not find any articles on other possible epigenetic changes in VSCC or its precursors, such as chromatin remodelling or histone modifications. Most research on hypermethylation has studied different genes so a comparison cannot be made. Only *CDKN2A* has been investigated by more than one group. The hypermethylation frequencies that were found differ greatly between LS, VIN, and VSCC. The trend appears to be more hypermethylation in VSCC, but with the limited data it is difficult to draw any conclusions. With the fast development of research techniques focusing on epigenetic alterations in tumours, and the knowledge already gained on targeted therapy for epigenetically altered tumours, future research on this topic is promising.

In conclusion, genetic and epigenetic changes are detected more often with increasing precursor and tumour stage, and are more frequently found in HPV-negative patients than HPV-positive patients. However, compared to other types of cancer, studies on genetic and epigenetic changes in vulvar cancer and its precursors is relatively few and, therefore, our knowledge on this subject is still limited. Most genetic studies focus on HPV infection and *TP53* mutations, , the latter being the most frequent genetic change found in human cancers so far. Recent studies provide evidence that somatic mutations often do occur in oth-

er genes, such as *CDKN2A* and *HRAS*. Of all premalignant and malignant vulvar lesions, HPV-independent VSCC represents the largest group of patients with the worst prognosis and most difficulties in the diagnosis and treatment of progressive tumours. The upcoming availability of screening methods for somatic mutations that provide information on the complete or very large parts of the genome, such as next generation sequencing, may provide us with more insight into the mutational and epigenetic landscape and the etiology of vulvar cancer. Hopefully, these advances will increase future treatment possibilities and improve prognosis.

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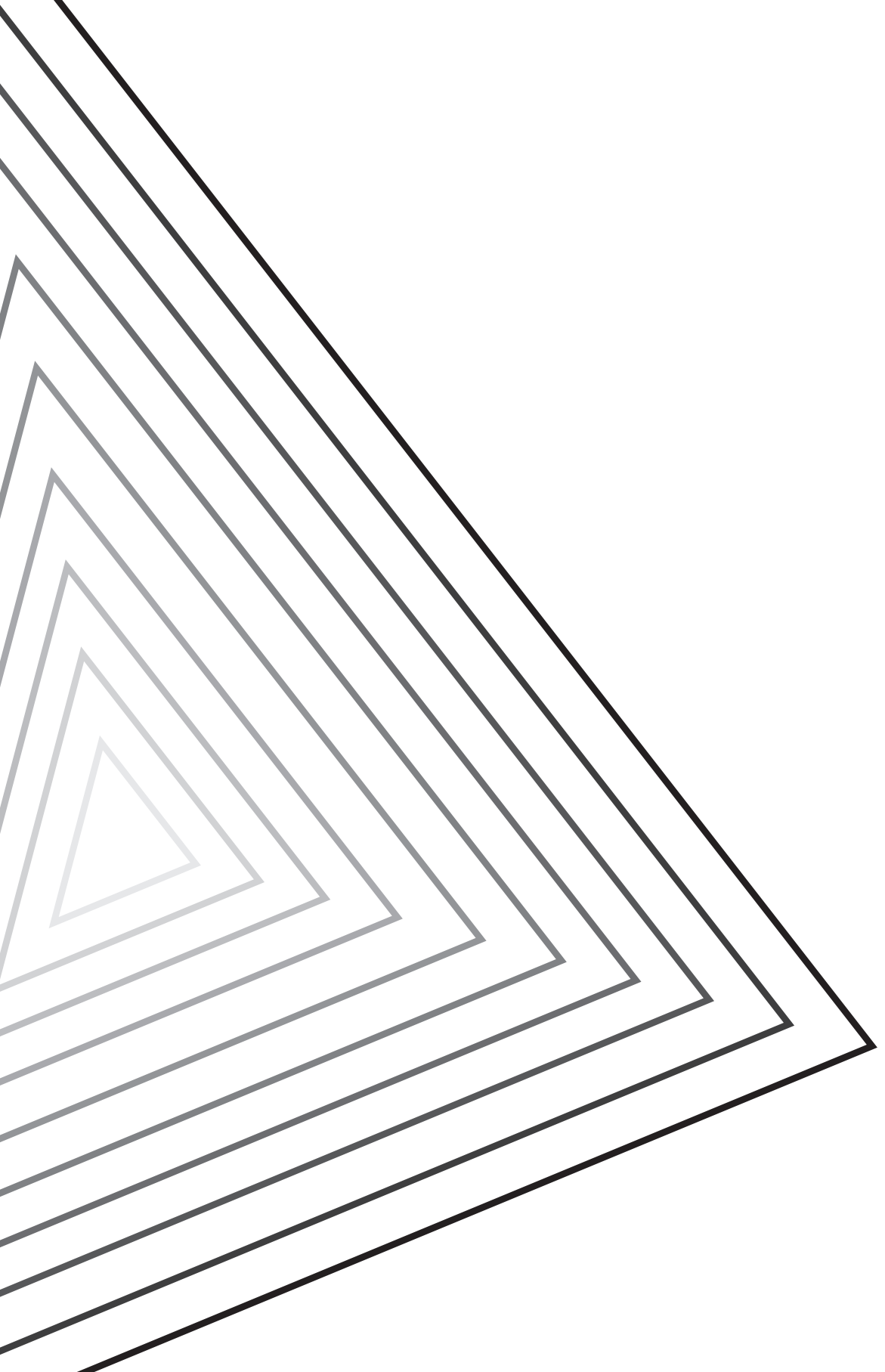

Chapter 5

Spindle cell morphology is related to poor prognosis in vulvar squamous cell carcinoma

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British Journal of Cancer (2013) 109, 2259–2265 | doi: 10.1038/bjc.2013.563

ABSTRACT

Background

Vulvar cancer is the fourth most common gynaecological malignancy, with an annual incidence of 2/100,000 women. Although most cases of early stage vulvar cancer have a good prognosis, recurrence and rapid tumour progression can occur. We investigated the prevalence of spindle cell morphology in vulvar cancer and its association with survival.

Methods

This retrospective cohort study included 108 patients with primary vulvar squamous cell carcinoma who were treated at the Leiden University Medical Center 2000–2009. Paraffin-embedded tissue was examined for the presence of spindle cell morphology. Survival and histology data were compared between cases with spindle and without spindle cell morphology.

Results

Twenty-two (20%) tumours showed spindle cells infiltrating the stromal tissue. All spindle cell tumours were human papillomavirus (HPV)-negative. Spindle cell morphology was strongly associated with poor prognosis and with a high risk of lymph node involvement at the time of diagnosis (relative risk 2.26 (95%CI 1.47-3.47)). Five-year disease-specific survival was lower in patients with vs. without spindle cell morphology (45.2% vs. 79.7%, respectively; P=0.00057).

Conclusion

Vulvar spindle cell morphology occurs frequently and seems to develop through the non-HPV pathway. It is associated with a worse prognosis than conventional vulvar squamous cell carcinoma.

Keywords

Vulva; carcinoma, squamous cell; carcinoma, spindle cell; prognosis

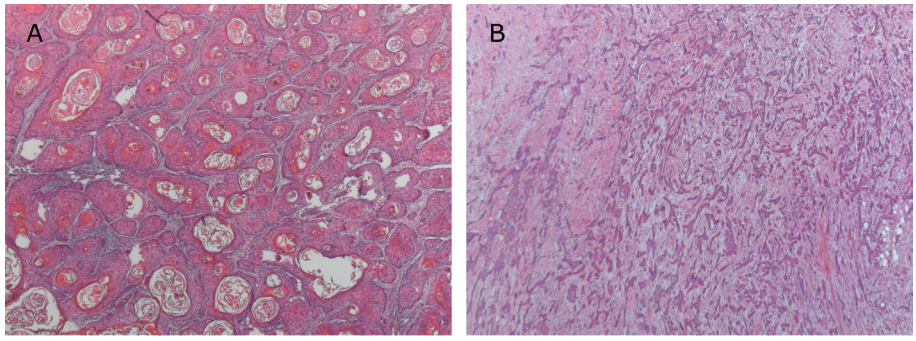
Introduction

Vulvar cancer is the fourth most common gynaecological malignancy in developed countries with an incidence of approximately 2 cases per 100,000 women each year (1-3). This cancer is mostly seen in post-menopausal women, and the average age at diagnosis is 70 years Nearly 90% of all vulvar carcinomas are vulvar squamous cell carcinoma (VSCC). (4). Squamous cell carcinomas of the vulva can be divided into two etiological types (5;6). The first type is seen mainly in younger patients and is associated with human papillomavirus (HPV) infection (7). The second type is seen mostly in elderly patients and seems to develop independent of HPV infection. This type of carcinoma is associated with lichen sclerosis and mutations of the *TP53* gene (7;8).

Vulvar carcinoma generally has a good prognosis when detected and treated at an early stage, but the most common treatment modalities, i.e. surgical removal and/or radiotherapy, can be mutilating and have high morbidity rates (9-11). A small proportion of patients suffer from early recurrence, rapid progression of tumour growth and death (12).Identifying risk factors for poor survival is important to further reduce the mortality and morbidity of vulvar cancer patients, but few studies have found new factors of clinical importance (13). To date, lymph node involvement is the only accurate prognostic factor for survival and recurrence (14).

A specific histological type of squamous cell carcinoma, termed spindle cell carcinoma, is seen occasionally. Spindle cell carcinoma is also referred to as pseudosarcoma or carcinosarcoma. Spindle cells are thin, slender, elongated cancer cells that infiltrate stromal tissue and that occur either as single cells or as cords of cells rather than as groups or islands (Figure 1). Spindle cell carcinoma is seen in many different types of cancer and is associated with a worse prognosis in cancers of the oral cavity, oesophagus and lung (15-19). Spindle-shaped epithelial cancer

Figure 1 *VSCC and spindle cell morphology*



*Representative images of vulvar squamous cell carcinoma (**A**) and vulvar squamous cell carcinoma with spindle cell morphology (20x magnification) (**B**).*

cells have lost their typical epithelial characteristics, and seem to gain the ability to infiltrate the underlying stroma and metastasize (20).

Spindle cell carcinomas consisting of spindle cells alone have been reported only incidentally in vulvar carcinoma, and it is thought to be an aggressive tumour type that occurs in 1% to 5% of all vulvar squamous cell carcinoma patients (15;21-27). Some VSCCs show a specific pattern of invasion in which there is infiltration of the stroma by single cells or cords of tumour cells adjacent to the 'conventional' squamous cell carcinoma cells. This pattern is also called 'spray pattern invasion'. This spray pattern invasion is seen more often than the rare vulvar spindle cell carcinoma and has been described before (28-30). A subpopulation of these vulvar carcinomas with a spray pattern of invasion clearly shows areas of infiltrating cells that resemble spindle cell carcinoma. We suggest that these tumours be termed, 'vulvar squamous cell carcinomas with spindle cell morphology', and describe the criteria used to characterize these tumours in the Methods section. The characteristics of tumours with this pattern of invasion and their association with survival have, to our knowledge, not been studied in a large cohort of patients. We hypothesise that tumours with spindle cell morphology, i.e. tumours consisting of both 'conventional' squamous cell carcinoma cells and spindle cells, may share some of the aetiology and clinical behaviour with the aggressive spindle cell carcinomas. We considered whether spindle cell morphology is a possible risk factor and studied its prevalence and possible relation with prognosis.

Materials and Methods

Patients

All patients who were primarily surgically treated for primary VSCC between 2000 and 2009 at the Leiden University Medical Center, a referral centre for gynaecological cancers in the Netherlands, were considered for inclusion in this study.

Clinical and follow-up data were retrieved from patient medical records and from the cancer registration database. Patients were excluded if they had received systemic immunosuppressive therapy, chemotherapy or radiotherapy in the pelvic area prior to surgery. Tumour staging was performed according to the FIGO system using histologically confirmed TNM data. We used the 1995 staging instead of the revised 2009 staging because of the retrospective design of the study (31;32). The patients were followed-up until 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.

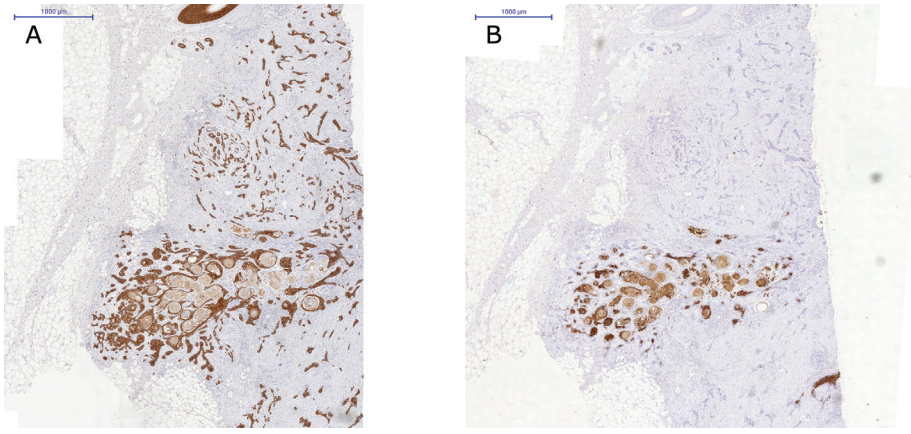
Microscopy and immunohistochemistry

All formalin-fixed, paraffin-embedded tissue blocks from the selected primary VSCC patients that were stored in the Leiden University Medical Center archives were examined microscopically for the presence of spindle-shaped tumour cells on haematoxylin and eosin-stained slides. Tumours from which only biopsies were taken were excluded from the study.

One series of consecutive 4-µm paraffin-embedded tissue sections was taken from all tumour-containing tissue blocks and stained as described previously (33) using primary antibodies for pankeratin AE1AE3 (mAB 3412, 1:2000, Millipore, Billerica, MA, United States of America), keratin 10 (DE-k10, 1:50, DAKO, Glostrup, Denmark) and keratin 14 (LL002, 1:2000, Abcam, Cambridge, United Kingdom). All slides were digitalized using a Mirax slide scanner and analysed using Panoramic Viewer (version 1.15.50, 3DHistec, Budapest, Hungary).

Since there is no established definition of spindle cell morphology, we categorized the samples as follows. Tumour cells were categorized as spindle cells if they had an elongated shape, infiltrated the underlying stromal tissue as single cells or as cords of cells instead of as islands of cells and were keratin 14-positive and keratin 10-negative as markers of dedifferentiation (Figure 2). When at least 10 spindle cells were seen per high power field using a 40 x 0.65 objective, independent of the proportion of spindle cells in relation to the solid component of the cancer, the tumour was categorized as having spindle cell morphology. Spindle-shaped cells in close proximity (<0.5 mm) to the solid component of the tumour were not marked as spindle cell carcinoma cells but were considered to be part of the spray pattern invasive border of the solid tumour.

Figure 2 *Keratin expression in VSCC with spindle cell morphology*



*A representative image of a sample of vulvar squamous cell carcinoma with spindle cell morphology stained for keratin 14 (**A**) and for keratin 10 (**B**). Note that the spindle-shaped cells are positive for keratin 14 but negative for keratin 10, while the solid component of the tumour is positive for both keratin 14 and 10 (Scale bar, 1000 µm).*

HPV analysis

DNA extracted from formalin-fixed, paraffin-embedded tumour tissue was used for HPV type analysis. To prevent and check for contamination, sections of a paraffin block without tissue, which were cut before each tumour sample, served as negative controls. All such controls were negative in the PCR analysis. The INNO-LiPA HPV Genotyping *Extra* Amp kit for in vitro diagnostic use (Innogenetics, Gent, Belgium), which is a highly sensitive hybridization assay, was used for HPV typing as described previously (34). This assay is able to detect oncogenic and common HPV types (34).

Statistical analysis

Statistical analyses were conducted using the IBM SPSS Statistics software package (version 20, IBM-SPSS Statistics, Armonk, NY, USA). The independent *t*-test was used to compare baseline variables, and Fisher's exact test was used to analyse categorical and normally distributed numerical data. The Shapiro-Wilk test was used to test for normality. The Mann-Whitney U test was used for data with a skewed distribution. Kaplan-Meier curves, the log rank test and Cox Proportional Hazard regression analysis were performed to analyse the differences in survival between the spindle and non-spindle groups. A P-value less than or equal to 0.05 was considered significant, corresponding to 95% confidence intervals. All tests were two-tailed. Results for normally distributed numerical data are presented as mean with standard deviation (SD), and results for skewed numerical data are presented as median with interquartile range (IQR).

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 the date of surgery until death from any cause or until the end of study follow-up. Disease-specific survival was measured from the date of surgery until death from vulvar cancer or until the end of study follow-up. Recurrent disease in the vulvar area was characterized as 'local recurrence', whereas recurrences in the inguinal region were characterized as 'regional recurrence'. Recurrent disease on the contralateral side of the vulva was considered to be a second primary tumour.

Results

Between January 2000 and December 2009, 129 patients were treated surgically 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 for another disease prior to the current diagnosis. Eleven patients were excluded because their tumours were biopsied but

no further surgical treatment was given. Tumour tissue from one patient was of poor quality and was not analysed further. Thus, a total of 108 patients with primary VSCC met all of our inclusion criteria. Table 1 lists the characteristics of these patients.

The median follow-up time was 39 months (IQR 16.3–70.5), and the mean age at diagnosis with vulvar carcinoma was 69.9 years (SD 14.1). Symptoms first

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

Characteristic			Value	
Follow up†	– mo		39.0	(16.3-70.5)
Age at diagnosis‡	– year		69.9	(14.1)
Duration of symptoms†	– mo		5.0	(2.0-18.0)
FIGO stage	– n	(%)		
Stage 1			30	(27.8)
Stage 2			36	(33.3)
Stage 3			30	(27.8)
Stage 4			12	(11.1)
Treatment	– n	(%)		
Radical vulvectomy			69	(63.9)
Radical local excision			39	(36.1)
Adjuvant radiotherapy			43	(39.8)
Adjuvant chemotherapy			1	(0.9)
HPV positive	– n	(%)	18	(16.7)
Lymph node metastases	– n	(%)	41	(38.0)
Unilateral			29	(26.9)
Bilateral			12	(11.1)
Extracapsular growth			17	(15.7)
Tumor size‡	– mm		32.1	(22.2)
Infiltration depth†	– mm		6.0	(4.0-11.0)
Vasoinvasion	– n	(%)	15	(13.9)
Lymphangioinvasion			3	(2.8)
Perineural growth			4	(3.7)
Positive resection margins			21	(19.4)
Disease status	– n	(%)		
Complete remission			83	(76.9)
Local recurrence			22	(20.4)
Second primary tumour			10	(9.3)
Regional recurrence			9	(8.3)
Regional metastases			8	(7.4)
Distant metastases			24	(22.2)
Died			59	(54.6)
Disease-specific death			28	(25.9)
5-year overall survival	– %	(SD)	51.9	(5.0)
5-year disease specific survival	– %	(SD)	72.9	(4.5)
5-year disease free survival	– %	(SD)	30.7	(4.9)

**Significant difference (P<0.05), †Median (interquartile range), ‡Mean (standard deviation).*
Abbreviations: N = number; mo = months; mm = millimetre.

occurred at a median of 5.0 months (IQR 2.0–18.0) before diagnosis. Microscopic evaluation showed a mean tumour size of 32.1 mm (SD 22.2) and a median infiltration depth of 6.0 mm (IQR 4.0–11.0). Lymph node metastases were found in 41 of the 108 patients (38.0%). Thirty patients (27.8%) had a tumour that was FIGO stage 1, 36 patients (33.9%) stage 2, 30 (27.8%) stage 3, and 12 (11.1%) stage 4. Forty-three patients (39.8%) received adjuvant radiotherapy to the groin and/or vulva; 38 of these patients received adjuvant radiotherapy because they were stage 3 or higher, and 5 of them received it because a tumour-free resection margin of less than 8 mm was obtained. One patient received adjuvant radiotherapy and chemotherapy because of close tumour-free resection margins plus the presence of multiple inguinal lymph node metastases.

At the endpoint of the study period, 20% of the patients had developed local recurrent disease and 26% had died from the disease, giving a 5-year disease-free survival of 31% (SD 4.9) and a 5-year disease-specific survival of 73% (SD 4.5).

Comparison of the clinical outcomes for patients with spindle and non-spindle cell morphology

Of the 108 tumours that were analysed, 22 tumours (20%) were identified as having spindle cell morphology and 86 (80%) as being non-spindle cell carcinomas. Table 2 summarizes the outcome measurements for patients with carcinomas with spindle cell and non-spindle cell morphology. The mean age did not differ between the two groups. Vulvar spindle cell carcinoma patients were diagnosed at a higher FIGO stage than non-spindle patients, though the duration of symptoms before diagnosis did not differ.

Forty-one of the 108 patients had lymph node metastases at the time of diagnosis, 15 of which were also positive for spindle cells. This gives a relative risk of lymph node metastasis in spindle cell morphology patients of 2.26 (95% CI 1.47–3.47) compared with patients without spindle cell morphology. Eighteen of 108 patients tested positive for HPV [type 16 (n=10), 18 (n=2), or 33 (n=5); one patient tested positive for both HPV types 16 and 33]. None of these patients had carcinomas with spindle cell morphology.

Compared to spindle cell morphology patients, more non-spindle patients achieved complete remission (83.7% vs. 50.0%, P=0.003). At the endpoint of the study period, 22.7% of the spindle cell morphology patients and 19.8% of the non-spindle cell patients had developed local recurrent disease, and 9.1% and 8.1% of patients, respectively, developed regional recurrences (P=1.000). In terms of disease-specific death, 50% of the spindle cell patients and 19.8% of the non-spindle cell patients died from the disease (P=0.002).

Overall survival differed significantly between patients with and without vulvar spindle cell morphology (Figure 3A), with 5-year overall survival of 27.3% (SD 9.5)

Table 2 *Comparison of the clinical outcomes for patients with spindle and non-spindle cell morphology.*

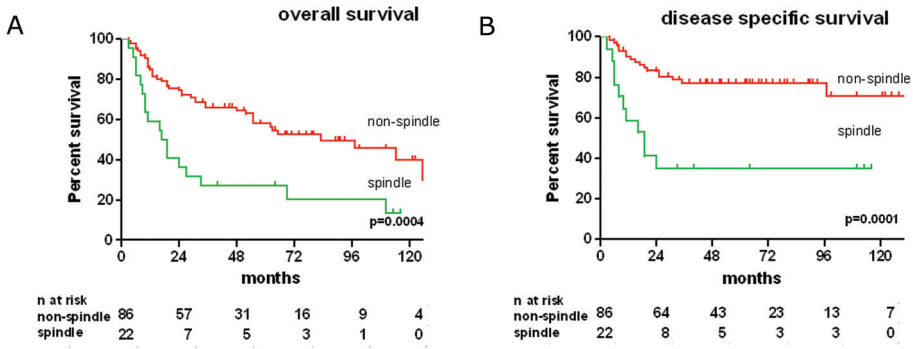
Outcome			Spindle n=22	(20.4%)	Non-spindle n=86	(79.6%)	P-value
Follow-up†	– mo		18.0	(8.8-46.0)	48.0	(123.0-77.5)	0.026*
Age at diagnosis‡	– yr		71.0	(13.2)	69.7	(14.4)	0.617
Duration of symptoms†	– mo		4.0	(2.8-12.5)	5.0	(2.0-22.0)	0.951
FIGO stage at first operation	– n	(%)					0.002*
Stage 1			3	(13.6)	27	(31.4)	
Stage 2			4	(18.2)	32	(37.2)	
Stage 3			8	(36.4)	22	(25.6)	
Stage 4			7	(31.8)	5	(5.8)	
Treatment	– n	(%)					
Radical vulvectomy			14	(63.6)	55	(64.0)	1.000
Radical local excision			8	(36.4)	31	(36.0)	
Adjuvant radiotherapy			14	(63.6)	29	(33.7)	0.020*
Adjuvant chemotherapy				(0.0)	1	(1.2)	
HPV positive	– n	(%)			18	(20.9)	0.011*
Lymph node metastases	– n	(%)	15	(68.2)	26	(30.2)	0.002*
Unilateral			8	(36.4)	21	(24.4)	0.001*
Bilateral			7	(31.8)	5	(5.8)	
Extracapsular growth			8	(36.4)	9	(10.5)	0.006*
Tumor size‡	– mm		38.7	(21.4)	30.5	(22.2)	0.610
Infiltration depth†	– mm		8.0	(4.5-12.0)	6.0	(3.0-11.0)	0.242
Vasoinvasion	– n	(%)	6	(27.3)	9	(8.3)	0.077
Lymfangioinvasion			1	(4.6)	2	(2.3)	0.499
Perineural growth			1	(4.6)	3	(3.5)	1.000
Positive resection margins			7	(31.8)	14	(16.3)	0.131
Disease status	– n	(%)					
Complete remission			11	(50.0)	72	(83.7)	0.003*
Local recurrence			5	(22.7)	17	(19.8)	0.771
Second primary tumour			2	(9.1)	8	(9.3)	1.000
Regional recurrence			2	(9.1)	7	(8.1)	1.000
Regional metastases			1	(4.5)	7	(8.1)	0.443
Distant metastases			7	(31.8)	17	(19.8)	
Died			18	(81.8)	41	(47.7)	0.007*
Disease specific death			11	(50.0)	17	(19.8)	0.002*
5-year overall survival	– %	(SD)	27.3	(9.5)	58.2	(5.6)	0.00041*
5-year disease specific survival	– %	(SD)	45.2	(11.4)	79.7	(4.6)	0.00057*
5-year disease free survival	– %	(SD)	25.0	(12..5)	44.3	(6.6)	0.149

**Significant difference (P<0.05), †Median (interquartile range), ‡Mean (standard deviation). Abbreviations: N = number; mo = months; mm = millimetre.*

vs. 58.2% (SD 5.6; P=0.00041), respectively. Disease-specific survival was significantly worse for spindle cell morphology patients than non-spindle cell morphology patients, with a 5-year disease-specific survival of 45.2% (SD 11.4) vs. 79.7% (SD 4.6; P=0.00057; Figure 3B). Five-year disease-free survival did not differ significantly between the two groups (25.0% vs. 44.3%, P=0.149).

Univariate Cox regression analysis for disease-specific death resulted in a hazard ratio of 3.50 (95% CI 1.63–7.52, P=0.001) for spindle cell morphology patients compared with non-spindle cell patients. After correction for the possible con-

Figure 3 *Survival curves*



*Overall survival (**A**) and disease-specific survival (**B**) in the 108 vulvar squamous cell carcinoma patients in this study. There were significant differences in both overall and disease-specific survival for patients that had carcinomas with vs. without spindle cell morphology (relative risk of dying 1.7 vs. 2.5; 95% CI 1.3–2.3 and 1.4–4.6; P=0.0043 and P=0.0041, respectively). Red is non-spindle, green is spindle.*

founders age and HPV infection, the hazard ratio became 2.71 (95% CI 1.26–5.81, P=0.011). When correcting for more tumour characteristics (tumour size, infiltration depth, positive resection margins), vulvar spindle cell morphology patients had a hazard ratio for disease-specific death of 2.51 (95% CI 1.12–5.64). Adding lymph node metastasis and regional or distant metastasis to the correction model gave a hazard ratio of 4.1 (95%CI 1.61–10.60) for patients with carcinomas with spindle cell morphology (Table 3).

Discussion

This study is, to our knowledge, the first to investigate vulvar spindle cell morphology in a large group of patients with VSCC. Spindle cell morphology occurred frequently in our series and was found in 20% of the VSCCs examined. Carcinoma with spindle cell morphology has not been reported as a separate entity in vulvar cancer prior to this report. A spray pattern of invasion, which also includes spindle cell morphology, has been reported in a few papers (28-30) and was found in 11 out of 26 VSCCs by Drew *et al* (29). In the latter study, which was relatively small, a spray pattern of invasion was significantly associated with poor survival after correction for FIGO stage alone.

Our study of a large cohort of VSCC patients shows that patients with spindle cell morphology have a worse prognosis than patients with conventional squamous cell carcinoma of the vulva. The disease-specific and overall survival was almost half that of non-spindle squamous cell carcinoma patients. After correcting for multiple possible confounders, vulvar spindle cell morphology remains an

Table 3 *Multivariate analysis of prognostic variables.*

		P-value	HR	95% CI for HR
Step 0	Spindle cell morphology	.001	3.503	1.632 - 7.517
		P-value	**HR**	**95% CI for HR**
Step 1	Spindle cell morphology	.011	2.706	1.262 - 5.805
	Age	.493	1.001	0.998 - 1.003
	HPV infection	.970	.000	N.A.
		P-value	**HR**	**95% CI for HR**
Step 2	Spindle cell morphology	.026	2.512	1.119 - 5.639
	Age	.850	1.000	0.997 - 1.002
	HPV infection	.971	.000	N.A.
	Tumour size	.205	1.016	0.991 - 1.042
	Infiltration depth	.906	1.004	0.940 - 1.072
	Positive resection margins	.335	1.734	0.566 - 5.306
		P-value	**HR**	**95% CI for HR**
Step 3	Spindle cell morphology	.003	4.135	1.613 - 10.601
	Age	.348	.999	0.996 - 1.001
	HPV infection	.964	.000	N.A.
	Tumour size	.050	1.028	1.000 - 1.057
	Infiltration depth	.562	.984	0.930 - 1.040
	Positive resection margins	.047	3.183	1.015 - 9.979
	Lymph node metastasis	.509	1.364	0.543 - 3.427
	Metastasis:			
	No metastasis	.000	Ref.	
	Locoregional metastasis	.001	14.807	3.204 - 68.425
	Distant metastasis	.000	39.734	11.341 - 139.214

Variables added in step 1: age, HPV. Variables added in step 2: tumour size, infiltration depth, positive resection margins. Variables added in step 3: lymph node metastases, infiltration depth, distant metastases.
Abbreviations: HR = hazard ratio

independent prognostic factor. None of the patients with spindle cell morphology showed HPV infection, strongly suggesting that these tumours arise through the non-HPV pathway (8;35).

The vulvar spindle cell morphology patients reported having symptoms for a similar length of time prior to diagnosis than non-spindle cell patients, but vulvar spindle cell carcinoma patients presented with higher FIGO stages, possibly because of the aggressive character of spindle cells and the rapid progression of this type of tumour.

Although this series is the largest reported in the literature, the number of patients in this study is still too small to correct for all potential confounders. However, even after correcting for multiple tumour characteristics that are likely to be part of the causal path (tumour size, infiltration depth, positive resection margins, lymph

node metastasis and distant metastasis), vulvar spindle cell morphology patients had a hazard ratio for disease-specific death of 4.14 (95% CI 1.61–10.60) relative to non-spindle cell patients, suggesting that the poor prognosis of these patients is associated with spindle cell morphology and is independent of other tumour characteristics. Caution has to be taken when interpreting multivariate analysis in small cohorts of patients. Given the distinct differences in survival, we suggest that this retrospective study be replicated at other large institutions to determine the added prognostic value of spindle cell morphology in a prognostic model.

Regarding the survival and overall incidence of spindle cells, selection bias could have been introduced by our inclusion of patients who were treated primarily by surgery. Patients that were treated by excision biopsy alone were not selected for this series, and such patients usually have smaller tumours and a better prognosis. On the other hand, patients with inoperable tumours, and likely a worse prognosis, were also not selected, so patient selection cannot fully explain the differences in survival. Notably, correcting for tumour size did not change the finding that spindle cell morphology patients have a significantly worse prognosis.

These results may have implications for the clinical management of VSCC. Spindle cells can be detected by a trained pathologist and, if necessary, be visualized using commonly available keratin stains. Given our finding that spindle cell morphology does not affect the risk of local recurrent disease, the current advice to perform a local radical tumour resection with 8-mm tumour-free margins after fixation seems appropriate (36). However, the increased risk of lymph node metastasis and poorer prognosis could influence the choice to perform adjuvant chemotherapy or radiotherapy in vulvar spindle cell morphology patients.

Conclusion

VSCC with spindle cell morphology appears to be an aggressive tumour type. In this series of patients, this cancer type had a worse prognosis than conventional VSCC as well as an increased risk of lymph node metastases at the time of diagnosis.

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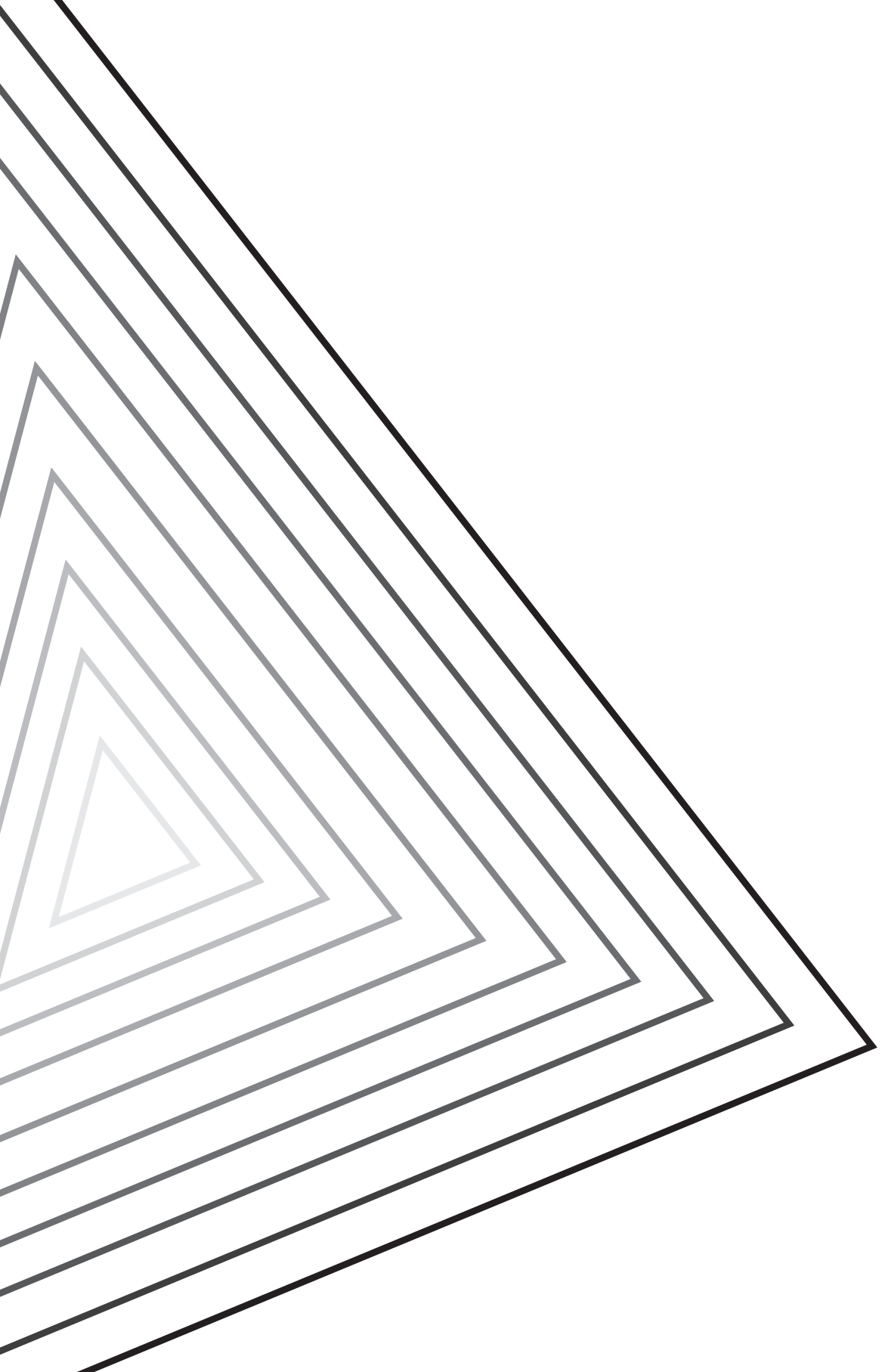

Chapter 6

Prognostic value and clinicopathologic characteristics of L1 cell adhesion molecule (L1CAM) in a large series of vulvar squamous cell carcinomas

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Oncotarget. 2016 May 3;7(18):26192-205. doi: 10.18632/oncotarget.8353

Abstract

Background

Vulvar cancer treatment is mostly curative, but also has high morbidity rates. In a search for markers that can identify patients at risk of metastases, we investigated the prognostic value of L1-cell adhesion molecule (L1CAM) in large series of vulvar squamous cell carcinomas (VSCCs). L1CAM promotes cell motility and is an emerging prognostic factor for metastasis in many cancer subtypes.

Methods

Paraffin-embedded tumour tissue from two cohorts (N=103 and 245) of primary VSCCs were stained for L1CAM, vimentin and E-cadherin. Patients of the first cohort were tested for human papilloma virus infection and sequenced for *TP53* and *CTNNB1* (β-catenin) mutations. The expression of L1CAM was correlated to clinical characteristics and patient survival.

Results

L1CAM expression was observed at the invasive front or in spray-patterned parts of 17% of the tumours. L1CAM-positive tumours expressed vimentin more often, but L1CAM expression was not associated with *TP53* or *CTNNB1* mutations. Five-year survival was worse for patients with L1CAM expression (overall survival 46.1% vs 63.6%, *P*=.014, disease specific survival 63.8% vs 80.0%, *P*=.018). Multivariate analysis indicates L1CAM expression as an independent prognostic marker (HR 2.9, 95% CI 1.10–7.68). An *in vitro* spheroid invasion assay showed decreased invasion of L1CAM-expressing VSCC spindle cells after treatment with L1CAM-neutralising antibodies.

Conclusion

This is the first study to show high L1CAM-expression at the infiltrating margin of VSCC's. L1CAM-expressing VSCCs had a significantly worse prognosis compared to L1CAM-negative tumours. The highest expression was observed in spindle-shaped cells, where it might be correlated to their invasive capacity.

Introduction

Vulvar cancer is the fourth most common gynaecological malignancy affecting approximately 2 in 100.000 women each year in developed countries (1;2). Vulvar cancer typically occurs in postmenopausal women: the mean age of diagnosis is 70 years (3;4). Two subgroups of vulvar squamous cell carcinoma (VSCC) are currently recognised. The first generally affects younger patients and is associated with infection by the human papilloma virus (HPV). The second develops independently from HPV infection and is associated with mutations in the *TP53* gene (5).

Patients diagnosed with vulvar cancer at an early stage generally have a good prognosis (90% 5-year survival for FIGO stage 1 patients) (3). However, some patients suffer from rapidly progressing tumours that often recur and metastasize. Surgical treatment of early stage vulvar cancer is curative in most cases, but unfortunately, it also results in high morbidity rates (6;7). Researchers have tried to find prognostic markers that can differentiate patients who require aggressive (surgical) treatment from patients who would benefit from a more conservative and less invasive approach. This can include less radical surgical margins or to waive lymph node dissection or sentinel node procedure (8). Despite these efforts, no prognostic markers are currently used in the clinical management of VSCC patients, except for lymph node metastasis, which is currently considered the most accurate predictor for prognosis (8;9).

L1 cell adhesion molecule (L1CAM, or CD171) is thought to be one of the many factors involved in the induction of Epithelial-to-Mesenchymal Transition (EMT), responsible for the gain of invasive properties of cancer cells. L1CAM is a membrane glycoprotein that plays a crucial role in neural development where it has a dual mechanism: it can either stimulate cell adhesion, or it can promote cell motility. In normal adult tissue, L1CAM is only expressed by nerve tissue, leukocytes and renal tubules of the kidney, whereas in cancer it has also been reported to be expressed on tumour cell surface (10-12). In tumour cells, L1CAM can switch from a cell adhesion to a cell motility promoting role, which is demonstrated by its stimulating effect on invasive growth of tumour cells (12;13). This is also illustrated by studies showing high L1CAM expression (sometimes even exclusively), at the invasive border of tumours (13;14). Finally, L1CAM can induce a more invasive phenotype in cell lines (15).

The prognostic significance of L1CAM expression has been addressed in many different types of cancer, including gynaecological cancers (14;16-23). Recently, two large studies showed the prognostic significance of L1CAM in low grade endometrioid endometrial cancers (24;25). L1CAM was found to be expressed in invasive areas of epithelial ovarian cancer and was correlated with poor clinical outcome and unfavourable clinicopathological features of the disease (21).

There are several hypothesis regarding the underlying mechanism of L1CAM upregulation in cancer. The three dominant hypotheses are that L1CAM is upregulated by mutant p53 (26), through Wnt-signalling (14;26) or through the induction of TGF-β family members (26;27). L1CAM expression has not been examined in vulvar cancer before, but some studies have reported a relation between morphological features of EMT and a worse survival in vulvar cancer (28;29).

In this study, we investigated the expression of L1CAM in a large series of 348 VSCC patients from two different academic hospitals and correlated it with survival. In order to further understand the process of L1CAM upregulation, clinicopathological characteristics and markers for EMT were studied in one of the cohorts. Finally, in a pilot in vitro study we have examined the role of L1CAM in invasion of vulvar cancer cells.

Methods

Patient selection and sample collection

All patients 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)

Two cohorts of patients from two different referral cancer centres were included in this study. The first cohort exists of 108 patients with primary vulvar squamous cell carcinoma who were surgically treated at the Leiden University Medical Center between 2000 and 2009. Of these patients, 5 tissue blocks did not contain sufficient tumour tissue anymore and were excluded, therefore resulting in a cohort of 103 samples. Patients were also excluded if they had received chemotherapy or radiotherapy in the pelvic area prior to the operation, or if they had received systemic immunosuppressive therapy (n=9). Patients who underwent excision biopsies without further surgery were excluded, because biopsies do not contain enough tumour material (n=11). Clinical and follow-up data were retrospectively retrieved from patient medical records and the institutional cancer registration database. Follow up ended in December 2012.

The second cohort consists of 298 patients with primary vulvar squamous cell carcinoma who were surgically treated at the University Medical Center Groningen between 1981 and 2001. Since 1984, clinicopathological and follow-up data of all patients referred to the Department of Gynecologic Oncology of the University Medical Center Groningen, the Netherlands are prospectively collected during

standard treatment and follow-up. All consecutive vulvar squamous cell cancer patients with T1-2 tumors were selected. Twenty-five patients were excluded because they did not undergo inuinofemoral lymphadenectomy (which was a selection criterium in the original study (30)). In 18 cases this was because of general bad health, in the other 7 cases because of FIGO stage 1A disease. With the tissue samples of these patients, a tissue micro array (TMA) was built as previously described (30). Therefore, this cohort will be referred to as the 'TMA cohort'. Patients were excluded when they had been treated with preoperative radiotherapy. Fiftythree patients were removed from analysis because too many cores were missing from the TMA, resulting in a cohort of 245 patients in total.

Tumour staging for both cohorts was performed according to the FIGO system; the 1995 staging instead of the revised 2009 staging was used because of the retrospective design of the study (31;32). In the multivariate survival analysis, we corrected for lymph node metastasis and tumour size instead of FIGO stage, because these factors are not subject to revisions of staging systems over time. These cohorts have been described before (29;30;33).

Immunohistochemistry

Formalin fixed, paraffin embedded (FFPE) tissue blocks were collected for all included patients. 4µm sections were cut and sections were stained with haematoxylin and eosin to select representative tumour containing tissue blocks and areas.

The selected sections were stained using anti L1CAM antibody clone 14.1, 1:500 (Covance, Princeton, NJ, USA) and counterstained with haematoxylin as described before (34). Stained sections were analysed by one PhD-candidate and one gynaecopathologist (MDT and TB) separately, blinded for patient characteristics and outcome data. L1CAM expression was marked "positive" if 5% or more of the tumour cells stained moderate or strong for L1CAM. All other staining patterns were grouped "L1CAM negative", which included 1) completely negative tumours with positive internal control, 2) tumours with scattered positive tumour cells (<5%) or 3) very weak stained tumours (intensity was compared with internal control).

L1CAM staining of nerve axons was used as an internal positive control. A consensus was reached for all samples.

The Leiden cohort was also stained for β-catenin antibody clone 14 1:800, e-cadherin C20820 1:100 (both BD Biosciences, Franklin Lakes, NJ, USA) and vimentin V9-2B 1:50 (Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands) according to the manufacturers protocol.

The TMA cohort consisted of 4 Tissue Micro Arrays (TMA) with tumour samples of 298 squamous cell vulvar cancer patients (30). For the TMA cohort, 4 µm sections of the TMA were cut. Samples on the TMA were scored for each tissue core separately, later combining the results from the three cores per patient. A patient

with at least one core with moderate to strong L1CAM expression was scored positive for L1CAM, and negative for L1CAM if at least two cores were present and all negative. If two or more cores were missing or missing for more than 50%, and the remaining core was not scored as positive, the patient was marked as missing and removed from further analysis.

Tumour cell isolation and spheroid invasion assay

From fresh residual tumour tissue (81 year old patient, FIGO stage IVa) collected after diagnostic use, tumour cells were isolated. Keratin staining of the tumour showed both solid and spindle shaped tumour cells (supplementary figure 1). The tumour was tested negative for HPV as described before (29). Cells were harvested after overnight incubation at room temperature in 5 ml DMEM (Invitrogen, United Kingdom) containing 1 mg/ml collagenase and 1 mg/ml dispase. Next day, cells were washed and subsequently incubated in RPMI 1640 containing 10% Fetal Calf Serum (F7524, Sigma-Aldrich, USA) 50 U penicillin per ml and 50 µg streptomycin per ml (G1397, Sigma-Aldrich, USA. During cell culture cobble shaped and spindle shaped tumour cells were identified and were separated. Both cell types were cultured further until pure populations were obtained. Supplementary figure 2 show the morphologic features of the cells. To characterise the cells *TP53* Sanger sequencing was performed, showing a *TP53* R248Q mutation identical to the original tumour both in the spindle shaped and the cobble shaped cell population, reaffirming the epithelial origin of both cell types. L1CAM expression on both cell types was evaluated by western blot analysis as described before (35) using mouse anti- L1CAM antibodies (clone L1-9.3/2a, 2.3 µg/ml in TBST) and chemoluminescent detection.

The spheroid invasion assay was performed as described before (36). In short, spindle and cobble VSSCs were grown to spheroids (500 cells per spheroid) by plating them on agarose-coated 96 well plates. After 48h spheroids were harvested and embedded in a collagen type-I matrix in the presence of 40 µg/ml isotype control or L1CAM neutralising antibodies (clone L1-9.3), both kindly provided by Prof. Dr. Altevogt (37). Invasion of the cells into the collagen matrix was analysed by microscopy and pictures were taken at 1 day after embedding at 10x magnification (Olympus microscope). At least 10 spheroids per conditions were analysed and the experiment was repeated two times.

HPV and mutation analysis

Mutation analysis and HPV typing were performed on the study cohort of 103 patients. The pancytokeratin-stained slides were used to select an area consisting of at least 70% tumour 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) (38). 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).

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 (39). This assay can detect oncogenic and common HPV types.

For analysis of somatic mutations in the *TP53* gene, DNA sequencing was performed for exons 5–8 as described before (33). Mutation genotyping of *CTNNB1* was performed using the GynCarta 2.0 panel (40), which covers 88% of the currently known *CTNNB1* mutations.

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 analyse categorical and normally distributed numerical data. 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 were performed to analyse differences in survival between groups of patients with and without L1CAM expression. 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.

Results

From the Leiden cohort, 103 patients were included, and tumour sections from all patients were analysed for L1CAM. The average age at diagnosis was 71 years and the mean follow-up time was 4 years. Table 1 lists the characteristics of all included patients for the study cohort.

Of the 103 patients in the study cohort, 16 (16%) were positive for L1CAM (table 2). Figure 1 shows an example of an L1CAM positive tumour. All moderate to strong expressing cells were found at the invasive border of the tumours or areas with pronounced spindle-cell morphology (figure 1). None of the more differentiated or solid, keratinizing tumours showed any L1CAM positivity. HPV was detected

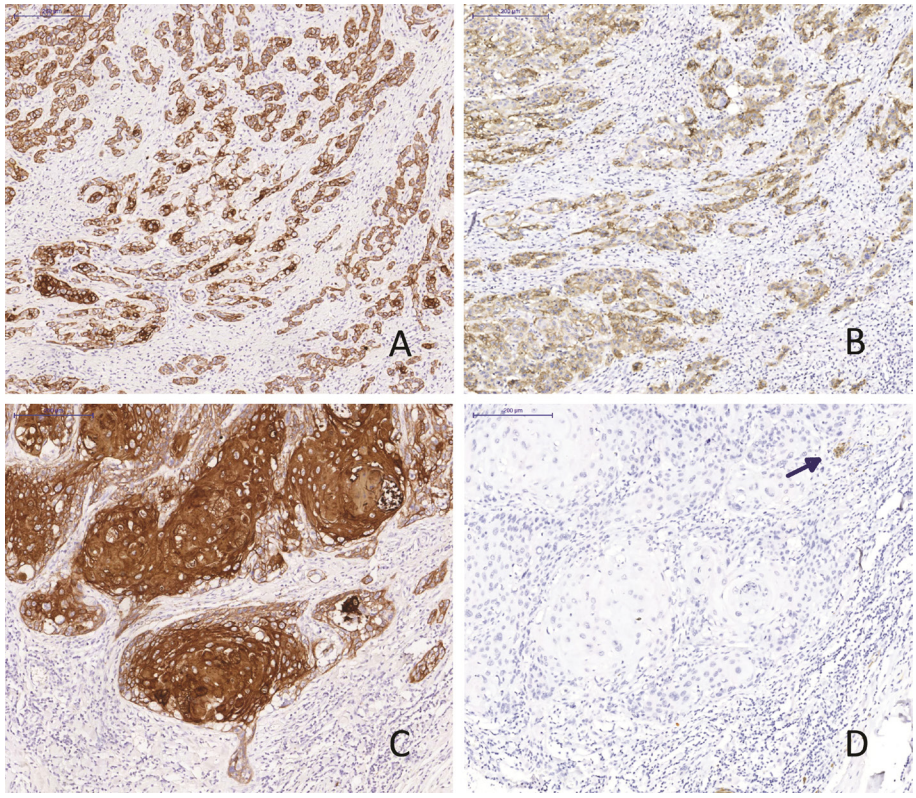
Table 1 *Patients characteristics of the Leiden cohort (n=103)*

Characteristic			value	
Follow up‡	– mo	(SD)	48.7	(36.1)
Age at diagnosis‡	– year	(SD)	70.7	(13.6)
Duration of symptoms †	– mo	(IQR)	5.0	(2.0-17.3)
FIGO stage	– n	(%)		
Stage 1			27	(26)
Stage 2			36	(35)
Stage 3			29	(28)
Stage 4			11	(11)
Lymph node metastases	– n	(%)	39	(38)
Extracapsular growth	– n	(%)	17	(17)
Tumor size‡	– mm	(SD)	31.8	(21.7)
Infiltration depth†	– mm	(IQR)	6.0	(4.0-10.0)
Positive resection margins			21	(20.4)
Disease status	– n	(%)		
Complete remission			80	(78)
Local recurrence			20	(19)
Regional recurrence			9	(9)
Died			56	(54)
Disease specific death			25	(24)
5-yr overall survival	– %	(SD)	52.5	(5.1)
5-yr disease specific survival	– %	(SD)	74.7	(4.5)
5-yr disease free survival	– %	(SD)	30.0	(5.0)

Table 2 *Comparison of patient characteristics for L1CAM positive and negative tumours in the Leiden cohort*

Outcome			L1CAM positive		L1CAM negative		p-value
			n=16	(16%)	n=87	(84%)	
Follow up‡	– mo	(SD)	29.0	(34.5)	52.3	(35.4)	0.017*
Age at diagnosis‡	– yr	(SD)	70.5	(13.1)	70.8	(13.8)	0.939
Duration of symptoms†	– mo	(IQR)	4.0	(2.3 - 73.5)	5.0	(2.0 - 14.8)	0.573
FIGO stage	– n	(%)					0.023*
stage 1			2	(13)	25	(29)	0.227
stage 2			6	(38)	30	(35)	0.784
stage 3			3	(19)	26	(30)	0.547
stage 4			5	(31)	6	(7)	0.013*
Lymph node metastases	– n	(%)	8	(50)	31	(36)	0.401
Extracapsular growth			6	(38)	11	(13)	0.024*
Tumor size‡	– mm	(SD)	39.5	(19.2)	30.6	(21.9)	0.156
Infiltration depth†	– mm	(IQR)	8.0	(5.5 - 13.3)	6.0	(3.5 - 9.0)	0.145
Positive resection margins			7	(43.8)	14	(16.1)	0.019*
Disease status	– n	(%)					
Complete remission			9	(56)	71	(82)	0.045*
Local recurrence			2	(13)	18	(21)	0.771
Regional recurrence			0	(0)	9	(10)	
Died			13	(81)	43	(49)	0.027*
Disease specific death			7	(64)	18	(24)	0.012*
5-yr Overall survival	– %	(SD)	18.8	(10)	58.7	(6)	0.001*
5-yr Disease specific survival	– %	(SD)	42.8	(15)	79.3	(5)	0.013*
5-yr Disease free survival	– %	(SD)	30.0	(15)	41.6	(7)	0.266

Figure 1 *L1CAM expression*



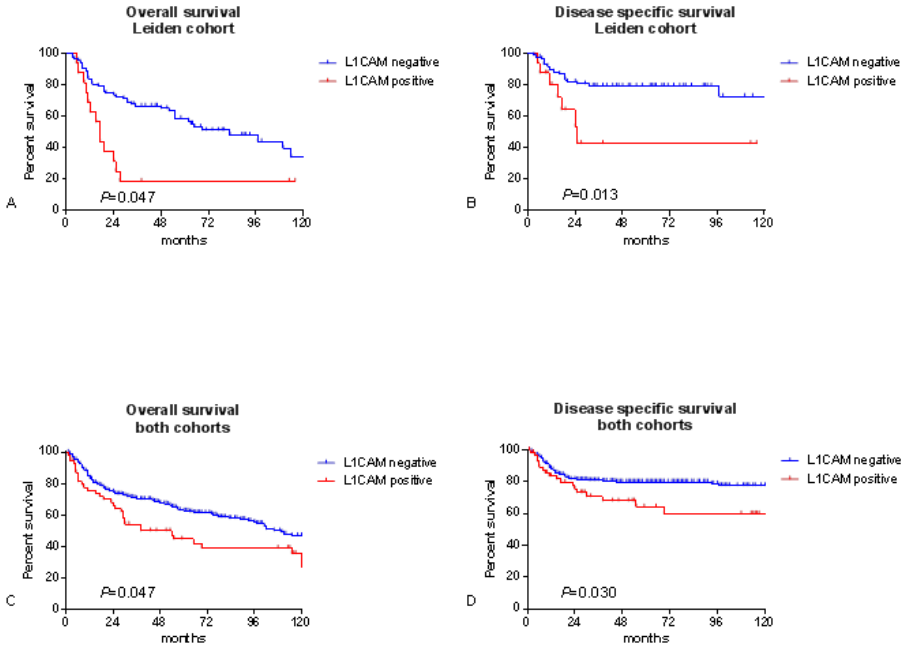
Two vulvar squamous cell carcinomas with spindle cell morphology (A and B) and solid growth pattern (C and D) stained for keratin (A and C) and L1CAM (B and D). Arrowhead: nerve axon as an internal positive control.

in 17 out of the total 103 patients (17%) and *TP53* mutations in 56 patients (54%). Previous research from our group has shown that VSCC with spindle cell morphology were more likely to carry *TP53* mutations and that spindle cell morphology was exclusively found in HPV negative patients (29). Although L1CAM expression was more frequently seen in spindle patterned tumours, there was no relation between L1CAM upregulation and *TP53* mutations and/or HPV infection. No *CTNNB1* mutations or aberrant nuclear β-catenin expression were detected in any of the samples. L1CAM upregulation was not associated with changes in e-cadherin expression, since all tumours express e-cadherin. Vimentin expression in the tumour was detected in 29 samples (28.2%) and was correlated to L1CAM expression (Spearman's rho 0.349, *P*=0.001) (table 3). An example of vimentin and L1CAM expression at the invasive border of a tumour is shown in supplementary figure 3.

Table 3 *Correlating molecular markers to L1CAM upregulation in the Leiden cohort*

Outcome			L1CAM positive		L1CAM negative		χ^2 p-value	Spearman Correlation	p-value
			n=16	(16%)	n=87	(84%)			
HPV positive	– n	(%)	1	(12)	16	(18)	0.462	-0.118	0.233
TP53 mutation	– n	(%)	10	(63)	46	(53)	0.278	0.070	0.482
HPV and/or TP53	– n	(%)	11	(69)	59	(68)	0.595	0.007	0.942
Vimentin	– n	(%)	10	(67)	17	(24)	0.004	0.349	0.001*

Figure 2 *Survival curves*



Kaplan Meier survival curves for the Leiden cohort, n=103 (A, B), and both cohorts combined, n=348 (C, D). P-values for log-rank test.

Comparison of survival data

Clinical data from the Leiden cohort were compared for patients with and without L1CAM positive tumours and listed in table 2. Patients with L1CAM positive tumours presented more often at the highest FIGO stage (31.1% vs 6.9%, *P*=.023) and if they had lymph node metastasis, it was more likely to be bilateral (31.3% vs 8.0%, *P*=.029) and with extracapsular growth (37.5% vs 12.6%, *P*=0.024). Patients without L1CAM staining were more likely to reach complete remission (81.6% vs 56.2%, *P*=.045). L1CAM

Figure 3 *Spheroid invasion assay*

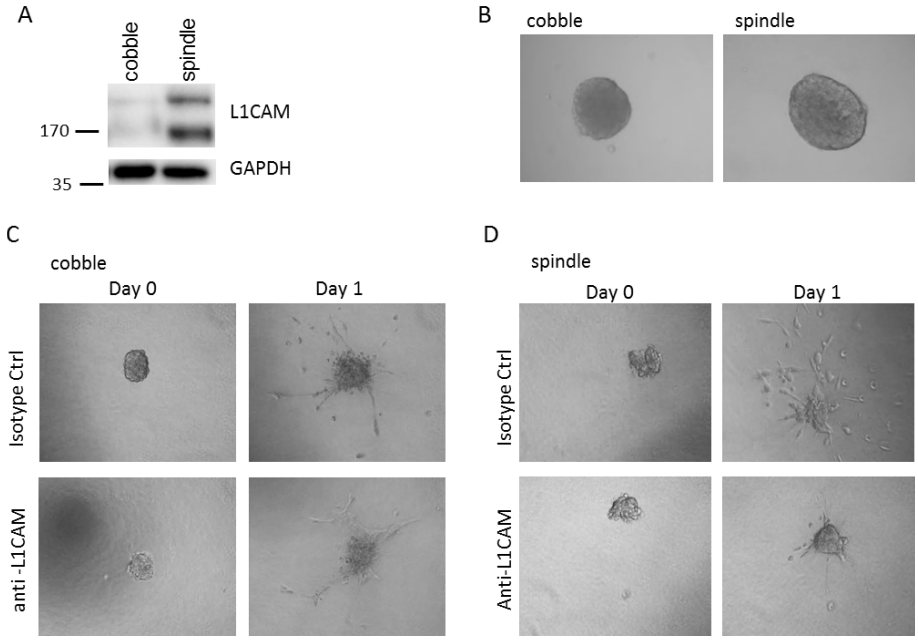


Figure 3A:Spindle shaped cells highly express L1CAM, while L1CAM expression on cobble shaped cells is very low. Figure 3B: Spindle and cobble cells form spheroids equally well and invade when embedded in a collagen matrix (C/D). Note that the invasion of the non-L1CAM expressing cobble shaped cells (Figure 3C) is hardly affected by L1CAM neutralising antibodies, while spindle cell invasion can be strongly inhibited by the L1CAM neutralising antibodies (Figure 3D).

positive patients had a worse 5-year overall and disease specific survival (18.8% vs 58.7%, log rank *P*=.001 and 42.8% vs 79.3%, log rank *P*=.013, respectively) (figure 2).

Patients with L1CAM-expressing tumours had a 2.9 times higher risk of dying from their cancer than patients lacking L1CAM expression (HR 2.9, 95% CI 1.10 – 7.68) when corrected for the possible confounders lymph node metastasis, tumour size and *TP53* mutations. Also for overall survival, L1CAM remained to be an independent prognostic factor when corrected for these confounders (HR 2.28, 95% CI 1.13 – 4.57) (Table 4).

Validating prognostic data in an independent cohort

Of the 245 patients in the independent TMA cohort, the average age at diagnosis was 72 years and the mean follow-up time was 5 years. Table 5 lists the characteristics of all included patients in the TMA cohort.

Of these 245 patients, 44 (18%) overexpressed L1CAM (table 6). The five year

Table 4 *Multivariate Cox regression analysis for the Leiden cohort (n=103)*

Disease specific survival	**variable**	**HR**	**95% CI**
	Lymph node metastasis	5.58	2.29 - 13.62
	Tumour size (mm)	1.02	1.00 - 1.04
	TP53 mutation	2.48	0.98 - 6.24
	L1CAM staining	2.91	1.10 - 7.68
Overall survival	**variable**	**HR**	**95% CI**
	Lymph node metastasis	3.33	1.91 - 5.78
	Tumour size (mm)	1.03	1.02 - 1.04
	TP53 mutation	1.50	0.84 - 2.67
	L1CAM staining	2.28	1.13 - 4.57

Table 5 *Patients characteristics of the TMA cohort (n=245)*

Characteristic	**n=245**		**value**	
Follow up‡	– mo	(SD)	60.0	(50.5)
Age at diagnosis‡	– year	(SD)	71.7	(13.1)
FIGO stage	– n	(%)		
Stage 1			51	(21)
Stage 2			94	(38)
Stage 3			67	(27)
Stage 4			33	(13)
Lymph node metastases	– n	(%)	92	(43)
Extracapsular growth	– n	(%)	40	(16)
Tumor size‡	– mm	(SD)	33.0	(17.2)
Infiltration depth†	– mm	(IQR)	7.0	(3.8-10.0)
Positive resection margins			21	(8.6)
Disease status	– n	(%)		
Complete remission			154	(63)
Local recurrence			50	(20)
Regional recurrence			14	(6)
Distant recurrence			6	(2)
Died			120	(49)
Disease specific death			50	(20)
5-yr Overall survival	– %	(SD)	59.6	(3.0)
5-yr Disease specific survival	– %	(SD)	78.2	(2.6)
5-yr Disease free survival	– %	(SD)	64.5	(3.0)

overall and disease specific survival was worse for patients with L1CAM expression, but it did not reach statistical significance (49.4% vs 61.3%, log rank *P*=0.074 and 70% vs 80%, log rank *P*= 0.159) (table 6). Since no material was available for mutation and HPV analysis, Cox regression analysis was performed correcting for lymph node metastasis and tumour size. Patients with L1CAM positive tumours showed a trend towards increased risk of dying, with a hazard ratio of 1.58 (95% CI 0.79 – 3.19) for disease specific survival and 1.48 (95% CI 0.93 – 2.35) for overall survival, these hazard ratios however did not reach statistical significance (supplementary table 1).

Table 6 *Comparison of patient characteristics for L1CAM positive and negative tumours in the TMA cohort*

Characteristic			L1CAM positive		L1CAM negative		p-value
			n=44	(18%)	n=201	(82%)	
Follow up‡	– mo	(SD)	55.2	(50.2)	61.9	(50.9)	0.432
Age at diagnosis‡	– yr	(SD)	73.1	(13.0)	71.2	(13.0)	0.381
FIGO stage	– n	(%)					0.031*
stage 1			7	(16)	44	(22)	
stage 2			13	(30)	81	(40	
stage 3			20	(46)	47	(23)	
stage 4			4	(9)	29	(14)	
Lymph node metastases	– n	(%)	21	(48)	71	(35)	0.104
Extracapsular growth			9	(21)	31	(15)	0.219
Tumor size‡	– mm	(SD)	35.8	(22.4)	32.8	(16.3)	0.303
Infiltration depth†	– mm	(IQR)	7.0	(4.0-10.0)	7.0	(3.5-10.2)	0.912
Lymfangio invasion	– n	(%)	11	(25)	28	(14)	0.112
Positive resection margins			5	(11)	16	(8)	0.386
Disease status	– n	(%)					
Complete remission			22	(50)	132	(66)	0.059
Local recurrence			9	(21)	36	(18)	0.413
Regional recurrence			2	(5)	10	(5)	
Distant recurrence			3	(7)	3	(1)	
Died			27	(61)	93	(46)	0.095
Disease specific death			12	(27)	38	(19)	0.219
5-yr Overall survival	– %	(SD)	49.4	(8.2)	61.3	(3.7)	0.074
5-yr Disease specific survival	– %	(SD)	70.4	(8.2)	80.3	(3.0)	0.159
5-yr Disease free survival	– %	(SD)	57.5	(9.4)	71.0	(3.6)	0.188

Table 7 *Comparison of patient characteristics for L1CAM positive and negative tumours in both the Leiden and the TMA cohort*

Characteristic			L1CAM positive		L1CAM negative		p-value
			n=60	(17%)	n=288	(83%)	
Follow up‡	– mo	(SD)	48.2	(47.7)	59.0	(46.9)	0.108
Age at diagnosis‡	– yr	(SD)	72.8	(13.0)	70.7	(13.3)	0.258
FIGO stage	– n	(%)					0.124
stage 1			9	(15)	69	(24)	
stage 2			19	(32)	111	(39)	
stage 3			23	(38)	73	(25)	
stage 4			9	(15)	35	(12)	
Lymph node metastases	– n	(%)	29	(48)	102	(35)	0.048*
Extracapsular growth			15	(25)	42	(15)	0.101
Tumor size‡	– mm	(SD)	36.7	(21.6)	32.1	(18.8)	0.092
Infiltration depth†	– mm	(IQR)	7.0	(4.4 - 11.0)	6.5	(3.5 - 10.0)	0.478
Positive resection margins			11	(18.3)	31	(10.8)	0.122
Disease status	– n	(%)					
Complete remission			31	(52)	203	(71)	0.006*
Local recurrence			13	(2)	58	(20)	
Regional recurrence			2	(3)	18	(6)	
Distant recurrence			4	(7)	10	(4)	
Died			40	(67)	136	(47)	0.007*
Disease specific death			19	(32)	56	(19)	0.023*
5-yr Overall survival	– %	(SD)	46.1	(7.2)	63.6	(3.0)	0.014*
5-yr Disease specific survival	– %	(SD)	63.8	(7.4)	80.0	(2.5)	0.018*
5-yr Disease free survival	– %	(SD)	57.5	(9.4)	71.0	(3.6)	0.188

Table 8 *Multivariate Cox regression analysis for both the Leiden and the TMA cohort (n=348)*

Disease specific survival	**variable**	**HR**	**95% CI**
	Lymph node metastasis	6.1	3.35 - 11.10
	Tumour size (mm)	1.02	1.01 - 1.03
	L1CAM staining	1.70	0.97 - 2.97
Overall survival	**variable**	**HR**	**95% CI**
	Lymph node metastasis	2.13	1.54 - 2.93
	Tumour size (mm)	1.02	1.02 - 1.03
	L1CAM staining	1.58	1.08 - 2.32

When taking all survival data together, thus creating a combined cohort of 348 patients (supplementary table 2) of which 60 (17%) were positive for L1CAM (table 7). The 5 year overall and disease specific survival was significantly worse for patients with L1CAM positive tumours (46.1% vs 63.6%, log rank *P*= 0.014 and 63.8% vs 80.0%, log rank *P*=0.018) (table 7). Patients with L1CAM positive tumours were more likely to have lymph node metastasis than patients without L1CAM expression (48.3% vs 35.4%, *P*=0.048) (table 7).

The multivariate Cox regression analysis for both cohorts combined, correcting for lymph node metastasis and tumour size provided a hazard ratio of 1.58 (95% CI 1.08 – 2.32) for overall survival and 1.70 (95% CI 0.97 – 2.97) for disease specific survival (table 8).

L1CAM inhibition decreases invasion of VSCC spindle shaped but not cobble shaped cells

To analyse the potential role for L1CAM in tumour cell invasion, we isolated spindle- and cobble-shaped cells from one VSCC which contained both components. Epithelial origin of the spindle- and cobble cells was confirmed by positive immunohistochemical stainings for pan-cytokeratin and presence of identical *TP53* mutations detected in the tumour from which the cells were derived (data not shown). L1CAM expression was analysed by western blot. Figure 3A shows that spindle shaped cells highly express L1CAM, whereas L1CAM expression on cobble shaped cells is very low. Next, both cell types were grown as spheroids and embedded in collagen type-I matrix to study the invasive properties (figure 3B). In the presence of an L1CAM neutralising antibody the invasion of the spindle cell population can be strongly inhibited, while the invasion of the non-L1CAM expressing cobble shaped cells is hardly affected (figure 3C and 3D). This experiment stresses the importance of L1CAM for the invasive potential of the spindle cells and opens possibilities to explore these antibodies in a therapeutic setting.

Discussion

L1CAM expression has shown to be a marker for poor disease outcome in several types of cancer (14;16-23) and this is study is the first to evaluate the prognostic capacity in vulvar cancer. Taking both the Leiden and the TMA cohort together, L1CAM was upregulated in 60 (17%) of all included VSCC. Patients with L1CAM expressing tumours have a significantly worse 5 year disease specific survival compared to patients with normal L1CAM expression (80% vs 64%). Furthermore our pilot in vitro data show an important role specifically for L1CAM in invasive properties of spindle shaped vulvar cancer cells.

There are three hypotheses how L1CAM is upregulated in cancer. Studies in colorectal cancer cell lines have shown that β-catenin (*CTNNB1*) mutations, and the subsequent aberrant activation of the Wnt signalling pathway, result in upregulation of L1CAM (14). Despite these findings in colorectal cancer, our work and that of others on vulvar cancers shows no evidence of *CTNNB1* gene mutations, nor nuclear β-catenin expression (28;33). Therefore, we do not expect that Wnt-signalling through β-catenin is a major factor in the upregulation of L1CAM in vulvar cancer.

P53 has also been postulated as a regulator of L1CAM expression and *TP53* is frequently mutated in vulvar cancer (26). In our current study, there was no correlation between *TP53* mutations and L1CAM positivity. Since HPV infection can alter the function of wild type p53, a relation between L1CAM expression and p53 aberration by *either* HPV infection or *TP53* mutation was investigated, but we did not find any correlation. We therefore concluded that p53 might not be driving L1CAM expression in vulvar cancer. An third alternative is that L1CAM expression is upregulated in the process of EMT (41;42). EMT-like changes have been described in vulvar cancer by us (29) and others (28) and was associated with poor clinical outcome. Our current findings show that L1CAM expression is exclusively found in areas with EMT-like growth (at the invasive border), or in tumours with predominant spray like growth, suggesting an association between EMT and L1CAM. Moreover, we confirmed that spindle shaped epithelial tumour cells highly express L1CAM protein, in contrast to their cobble shaped counterparts, which show very low or absent L1CAM.

Together, our findings indicate that L1CAM upregulation may be a consequence of EMT-like changes in vulvar cancer. Our spheroid invasion assay suggests that the invasion of these spindle shaped vulvar tumour cells can be strongly inhibited by treatment with L1CAM neutralising antibodies. These findings are is in line with other tumour models, where treatment with L1CAM neutralising antibodies seems to inhibit tumour growth and metastasis (37). Therefore our work contributes to the growing evidence showing efficacy of inhibiting tumour growth by L1CAM antibodies and stresses the need for further evaluating its therapeutic potential in vulvar and other cancers.

The strength of this study is the relatively large number of included patients with vulvar cancer. When combining the two cohorts, the statistical power of our analyses increases, which underlines the potential prognostic value of L1CAM expression in vulvar cancer. We do want to encourage other institutes to reproduce our study in order to validate our hypothesis. Because of the low incidence of vulvar cancer, we suggest these should be multicentre studies containing at least hundreds of patients.

A potential weakness of this study is its retrospective design. Since therapies change and improve over time, using samples dating back to 1984 might distort the survival analysis. Also, by excluding patients who underwent small excision biopsies and needed no further surgical excision, small sized tumours that have a relatively good prognosis might have been excluded. On the other hand, patients that did not receive surgical treatment were probably also excluded because of the large size or metastasis of their tumour. By including tumour size in the multivariate analysis, we have corrected for this possible bias. For the TMA cohort, only tissue micro arrays were available. Although three cores from different locations in the tumour were included for each patient, chances still are that L1CAM positive areas of the tumour were missed when taking these tissue cores. While the percentage of L1CAM positive samples was comparable between the study and the TMA cohort (16 and 18%), patients might have been allocated to the L1CAM negative group, thus diluting the effect L1CAM expression has on prognosis. The detected differences in survival are therefore on the safe side and might in fact be even more significant if full slides would have been available for this study.

In summary, we have shown for the first time that L1CAM is expressed in 17% of the VSCC's and that it is an independent prognostic factor for both overall and disease specific survival. Therefore L1CAM proves to have potential as a reliable biomarker that can be used to discriminate high risk from low risk vulvar cancer patients. We have studied and validated this prognostic significance in a large cohort of a relatively rare cancer type. Our results implicate that, unlike in other cancers, p53 and Wnt-signalling do not appear to play a dominant role in the (up) regulation of L1CAM. More likely, our data point towards a link between EMT and L1CAM expression in VSCC. We can conclude that L1CAM expression represents a promising prognostic biomarker in vulvar cancer. In addition, the potential to use L1CAM as a target for therapy based on our vulvar cancer cell invasion assays, warrants further investigation.

Acknowledgements

We thank Natalja ter Haar, Enno Dreef and Michelle Osse for their help in conducting the experiments, and we thank Katja Jordanova for the invitation of Zina Ivanova to our laboratory. This work was supported by the Alpe d'huZes/Bas Mulder award 2011 (UL20011-5051) to LH and RB. We thank Prof. Altevogt (German Cancer Research Centre, Heidelberg, Germany) for valuable discussions and providing the L1CAM neutralising antibodies.

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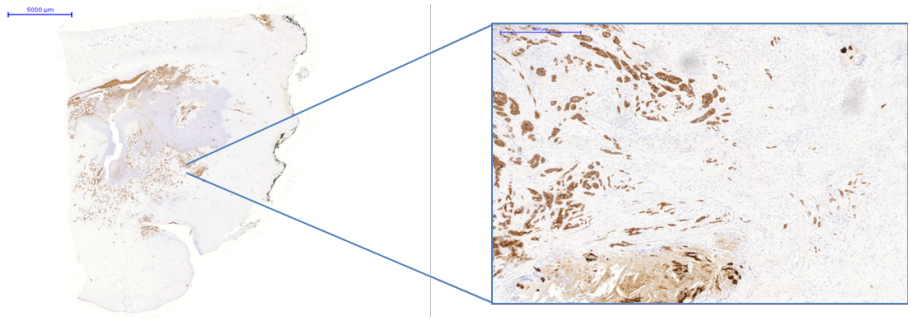
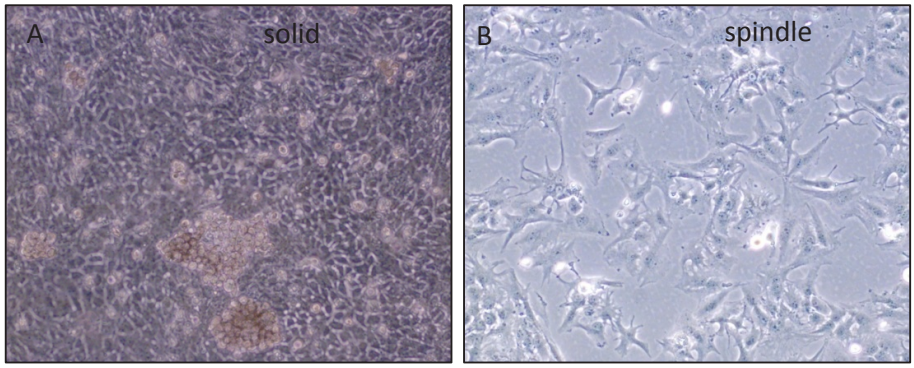
Supplementary tables and figures

Supplementary figure 1



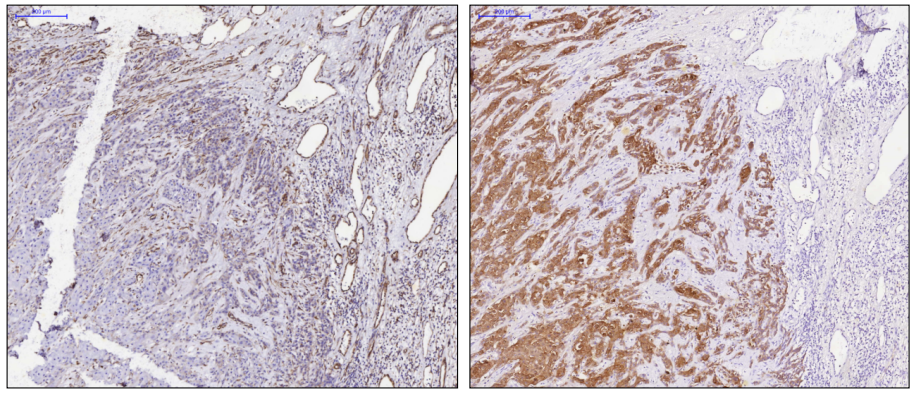
Original tumour from which two cell line populations were isolated, stained for pancytokeratin showing both solid an spindle shaped tumour cells.

Supplementary figure 2



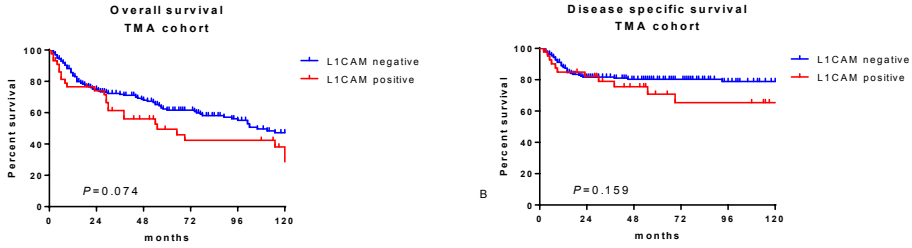
Bright light microscopy images of the two cell populations consisting of cobble shaped cells from the 'solid' tumour (A), and spindle shaped cells (B).s.

Supplementary figure 3



Vimentin and Keratin expression in the tumour border of a spindled vulvar cancer.

Supplementary figure 4



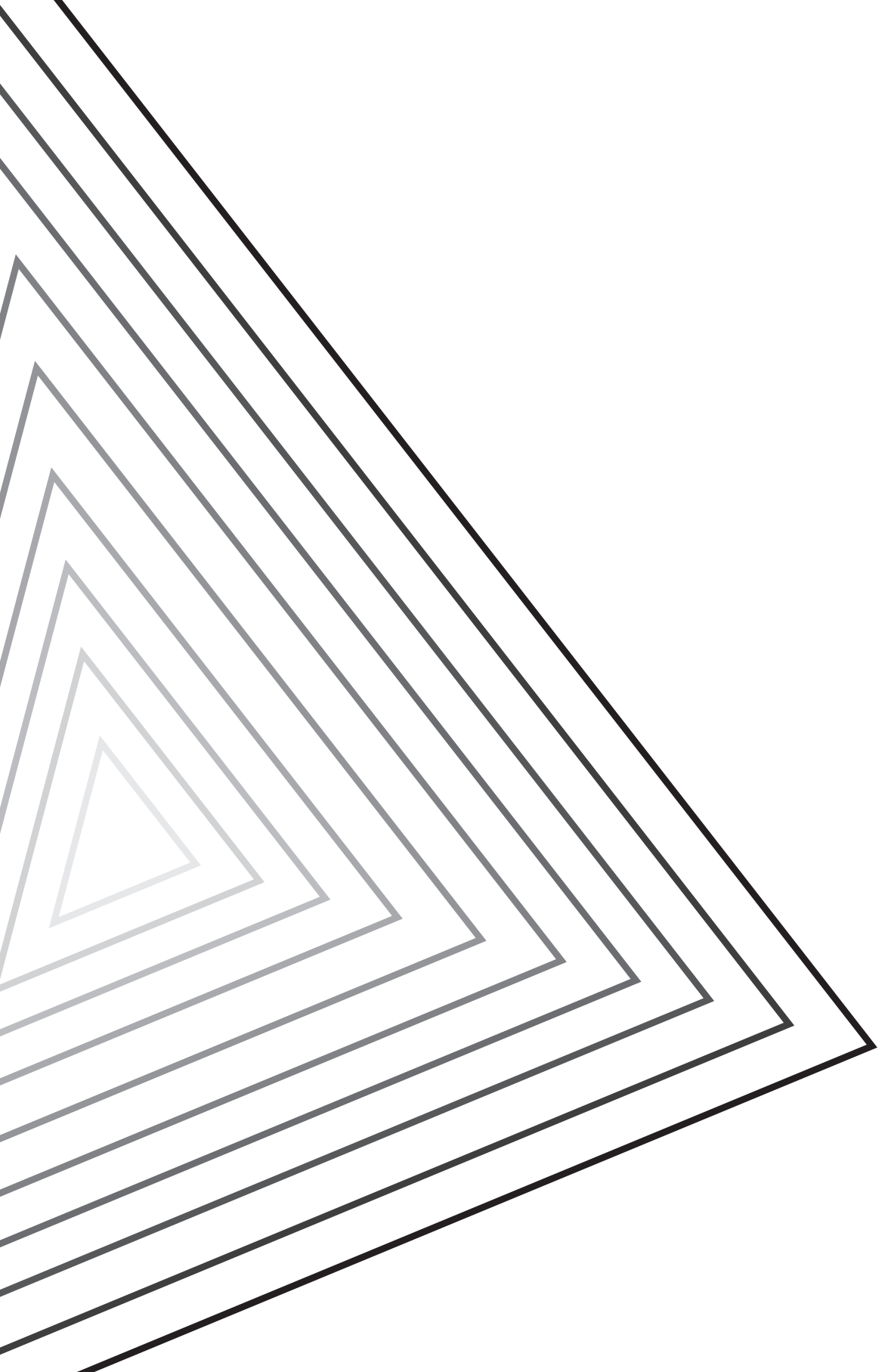
Overall and disease specific survival curves of the TMA cohort (N=245)

Supplementary table 1 *Multivariate Cox regression analysis for the TMA cohort (n=245)*

Disease specific survival	**variable**	**HR**	**95% CI**
	Lymph node metastasis	7.26	3.17 - 16.62
	Tumour size (mm)	1.02	1.01 - 1.04
	L1CAM staining	1.58	0.79 - 3.19
Overall survival	**variable**	**HR**	**95% CI**
	Lymph node metastasis	1.91	1.28 - 2.85
	Tumour size (mm)	1.02	1.01 - 1.03
	L1CAM staining	1.48	0.93 - 2.35

Supplementary table 2 *Patients characteristics of both the Leiden and the TMA cohort together (n=348)*

Characteristic	**n=348**		**value**	
Follow up‡	– mo	(SD)	57.1	(47.1)
Age at diagnosis‡	– year	(SD)	71.1	(13.2)
FIGO stage	– n	(%)		
Stage 1			78	(22)
Stage 2			130	(37)
Stage 3			96	(28)
Stage 4			44	(13)
Lymph node metastases	– n	(%)	131	(38)
Extracapsular growth	– n	(%)	57	(16)
Tumor size‡	– mm	(SD)	32.9	(18.8)
Infiltration depth†	– mm	(IQR)	7.0	(4.0 - 10.0)
Positive resection margins			42	(12.1)
Disease status	– n	(%)		
Complete remission			234	(67)
Local recurrence			71	(20)
Regional recurrence			20	(6)
Distant recurrence			14	(4)
Died			176	(51)
Disease specific death			75	(22)
5-yr Overall survival	– %	(SD)	60.4	(2.8)
5-yr Disease specific survival	– %	(SD)	77.5	(2.4)
5-yr Disease free survival	– %	(SD)	68.7	(3.4)



Chapter 7

Targeted sequencing of primary, metastatic and recurrent spindle cell variant vulvar cancer reveals a highly heterogeneous genetic disease

Marjolijn D Trietsch, Nalini Siewnath, Natalja T ter Haar, Willem E Corver, Gert Jan Fleuren

Abstract

Vulvar squamous cell carcinoma (VSCC) patients can suffer from rapid progression and recurrence. A higher risk of lymph node metastases and worse survival has been attributed to VSCCs with spindle cell morphology. To determine whether the spindle cell component is the origin of metastasis, we analyzed the mutational status of 66 primary VSCCs and their spindle cell components, lymph node metastases, and recurrences.

Mass spectrometry screening was performed for 13 relevant genes (*BRAF*, *CDKN2A*(p16), *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A* and *PTEN*). Solid components of VSCCs with and without spindle cell morphology were also screened for mutations in *TP53*.

TP53, *CDKN2A* and *HRAS* were most frequently mutated. Mutational profiles differed from the primary tumor in 64% of lymph node metastases and 81% of recurrences. There was no difference in mutation frequency between VSCCs with and without spindle cell morphology (77 vs 58%, NS). Spindle cell components were more likely to have the same profile as adjacent solid components (86% of cases).

Our study reveals that VSCCs likely consist of multiple, genetically different, clones. There is no proof of a selective role for somatic mutation(s) in the onset of spindle cell morphology. VSCCs with spindle cell morphology have a higher risk of lymph node metastasis and a worse 5-year survival. However, based on the mutational spectra, we have not been able to identify spindle cell components as the origin of metastasis or recurrence. Our findings could be of importance when developing targeted therapy.

Introduction

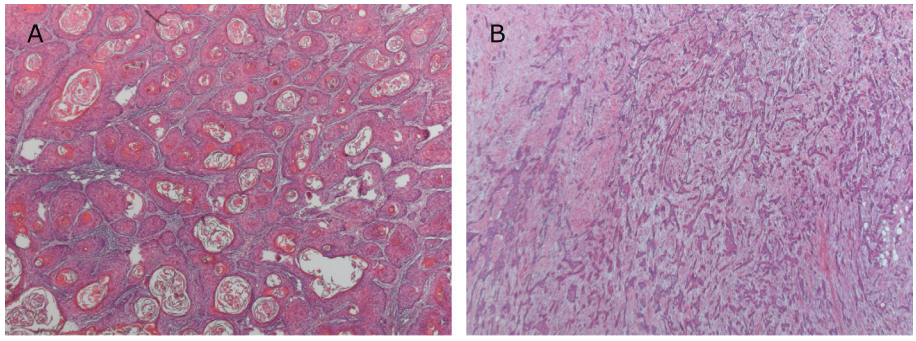
Vulvar cancer is a relatively rare gynecological malignancy, with approximately 2 new cases per 100,000 women each year (9;15;18;22). It is mostly seen in post-menopausal women, with an average age at diagnosis of 70 years. The most prevalent subtype of vulvar cancer is squamous cell carcinoma (VSCC), but other types such as basal cell carcinoma and melanoma also occur. VSCC is known to develop through two different etiologic pathways (8;34). The first pathway is clearly associated with infection by oncogenic high risk human papilloma viruses (HPV) and is mainly seen in younger patients. HPV produces proteins E6 and E7 that interact with the tumor suppressor proteins p53 and pRB (37), resulting in loss of wild-type function. The second pathway, which usually affects older patients, develops independent from HPV infection, and is associated with the chronic skin condition lichen sclerosus (3;33;34). HPV-negative VSCCs often carry somatic mutations in, amongst others, the *TP53* gene (30). The HPV dependent and the HPV independent pathway differ greatly in onset, but they do share a key role of p53.

Although surgical treatment is curative in most early stage cases, some patients suffer from rapid progression and recurrence (2;6;11) due to yet unknown reasons. In higher staged VSCC, radiotherapy and chemotherapy can be applied, but these treatment modalities have high morbidity rates (11). It is important to find risk factors that can help clinicians to identify those patients that are most at risk of progressive or recurrent disease, but to date the presence of lymph node metastases is the only accurate prognostic factor for survival and recurrence (12;20).

Approximately 20% of VSCCs have a subpopulation of cells with a distinct type of morphology called 'spindle cell morphology'. This subgroup of, almost exclusively HPV negative, VSCCs has a tumor component with loose, elongated tumor cells budding from the invasive front of the tumor. VSCCs with spindle cell morphology (VSCC-S) have a higher risk of lymph node metastasis and a worse 5-year survival when compared to VSCC that consist of a 'solid' component alone (31). (Figure 1)

The etiology of vulvar spindle cell morphology is not yet fully understood. Spindle shaped epithelial tumor cells seem to have lost part of their epithelial characteristics, and gain mesenchymal traits and metastatic capacities, known as epithelial-to-mesenchymal transition (EMT) (1;16). These metastatic capacities are reflected by the fact that VSCC-S have a higher risk of lymph node metastases (31). It is thought that changes in the TGFβ pathway might play a role in the onset of EMT and spindle cell morphology (26), but other molecular or genetic processes such as somatic mutations may also be of importance. Although it is a plausible hypothesis, it has not been proven that the spindle shaped cells are the origin of metastasis and recurrences, or that the tumor as a whole has gained aggressive traits.

Figure 1 *Vulvar squamous cell carcinoma with spindle cell morphology*



Representative images of vulvar squamous cell carcinoma (A) and vulvar squamous cell carcinoma with spindle cell morphology (20x magnification) (B).

In this study, we have compared the mutational profile of VSCCs with and without spindle cell morphology to gain insight in the recurrence and metastatic process of VSCC-S. We used the genetic alterations as identifiers to recognize a progressive pathway from solid primary tumor to spindle cells, which in term might be the origin of lymph node metastases and recurrences.

Methods

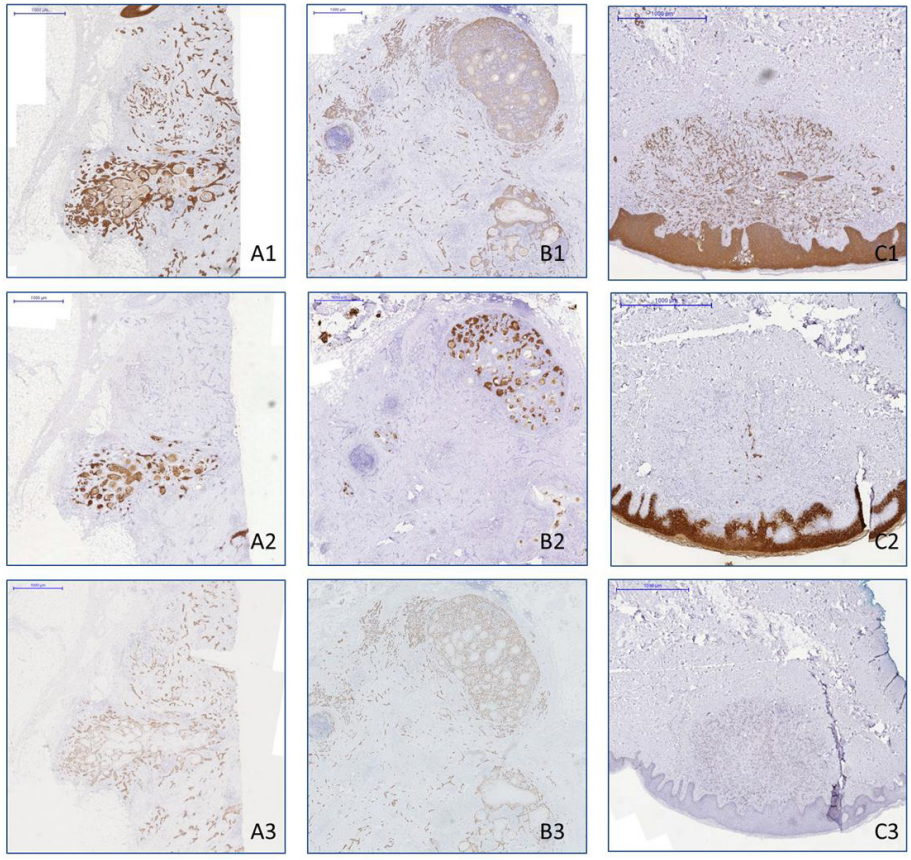
Patients

All patients that were surgically treated for primary VSCC in the Leiden University Medical Center between 2000 and 2010 were selected. All tissue samples were screened for mutations and spindle cell morphology before (31;32). Clinical follow up data were examined up to December 2012. Patients who were surgically treated for recurrence or lymph node metastasis within this follow up period were included in this study. Patient samples were handled according to the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies.

Identification of spindle cell morphology

All surgically removed recurrences and lymph nodes from the patients that were selected as described above, were screened for spindle cell morphology on all available tissue blocks. In short, 4 µm sections were cut from paraffin embedded, formalin fixed tumor tissue blocks, and stained for pankeratin, keratin 10 and keratin 14, respectively (27) clones AE1/AE3, MAB3412, 1:2000, Millipore, Billerica, Massa-

Figure 2 *Figure showing immunohistochemical staining of primary, lymph node and recurrence with spindle cell morphology*



Tissue taken from the primary tumor (A), the lymph node metastasis (B) and the recurrence (C) of the same vulvar squamous cell carcinoma patient, stained for pancytokeratin (1), cytokeratin 10 (2) and p53 (3). Note the presence of both spindle cell morphology and solid tumor in the lymph node metastasis. Spindle cells can be distinguished from 'normal' VSCC cells by the presence of pancytokeratin staining (1), and the absence of cytokeratin 10 staining (2). Slides 3 are stained for tp53 and show a mutational staining pattern in all three tissue specimens.

chusetts, USA; DE-K10, 1:50, DAKO Glostrup, Denmark; and LL002, 1:2000, Abcam, Cambridge, UK). Spindle cell morphology was determined as described before (31). In short: loose, elongated cells with a minimum distance of 0.5 mm to the solid component of the tumor and with a positive staining for pankeratin and keratin 14, but negative for keratin 10 were marked as spindle cells. Figure 2 shows an example of the expression of these proteins in a VSCC with spindle cell morphology.

DNA Isolation and FACS

The stained tissue slides were used to select and mark solid and spindle cell regions. The marked areas were transferred to the corresponding FFPE tissue blocks. Next, 4 - 5 0.6 mm diameter tissue cores were taken from the selected areas and transferred into a micro vessel.

DNA from solid tumor component tissue cores was isolated using a Tissue Preparation System (Siemens Healthcare Diagnostics, Malvern, Pennsylvania, USA) as described previously (14). The tissue cores where carefully punched from solid tumor tissue to prevent high amount of 'contamination' with stromal tissue.

Spindle cell morphology has a diffuse growth pattern in which spindle shaped cells are frequently intermingled with other types of tumor and normal infiltrating cells. This has consequences for further downstream genetic analysis using total DNA extracts. To circumvent this problem we used multiparameter fluorescence-activated cell sorting (FACS) to obtain pure tumor subpopulations using a dissociation protocol (4) with modifications that allowed the simultaneous analysis of pan-keratin, keratin 10, keratin 14, vimentin and DNA content.

Tissue cores from spindle cell morphology areas were cut into small pieces and were dewaxed using xylene, and were rehydrated. Next, tissues were subjected to heat-induced, antigen retrieval (HIAR) by heating the samples to 80°C in 10 mM sodium citrate buffer for 60 minutes. Samples were digested for 60-90 minutes at 37°C in a 0.1% mixture of collagenase and dispase in Rosswell Park Memorial medium (RPMI), with intermitted grinding at 15 minute intervals using the GentleMACS tissue grinder (protocol spleen 03_02)(Miltenyi Biotec, Bergisch Gladbach, Germany). When samples were sufficiently digested and the solution gained a cloudy appearance, samples were washed in PBA-Tween and filtered through an 80 µm mesh filter. Cell concentration was determined using the Muse Cell Analyzer (Merck Millipore, Darmstadt, Germany). 10^6 cells were incubated overnight at 4°C with 50 µL of primary antibody. Next day, samples were washed in PBA-Tween and incubated with a mixture of secondary goat anti-mouse subclass specific fluorescently labelled antibodies on ice for 60 minutes. Labelling concentrations and manufacturers are stated in Table 1. Cells were washed with NST buffer. (146 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM CaCl2, 0.5 mM MgSO4, 21 mM MgCl2, 0.05% bovine serum albumin, 0.2% Nonidet P40 (Sigma)) with 4,6-diamindino-2-phenylindole (DAPI; 10 µM (Sigma))For every 10^6 cells, 0.5mL (10 µM) of DAPI in NST buffer was incubated in the dark at 4°C for at least 2 hours.

Flow cytometric analysis was performed using an LSRII flow cytometer (BD Biosciences, San Jose, CA). A UV 355 nm, blue 488 nm and the red 635 nm laser were used for excitation. DAPI, FITC, R-PE, APC and APC-Cy7 fluorescence were collected using the following band pass filters, respectively: 450/50 nm, 530/30 nm, 572/25 nm, 670/14 nm and 780/60 nm. Diva 6.3.1 software was used for acquisition. Data files containing information from at least 50,000 single cell events were analyzed using ModFit 4.0, remotely linked to WinList 8.0 (Verity software House, Topsham, ME).

Table 1 *Antibody concentrations*

primary antibody	clone	isotype	concentration	company	secondary antibody	concentration	company
pankeratin	Ks pan 1-8	IgG2A	1:5	Acris Antibodies, Herford, Germany	APC	1:200	SBA, Birmingham, AL, USA
keratin 10	DE-K10	IgG1	1:5	DAKO, Glostrup, Denmark	FITC	1:100	SBA, Birmingham, AL, USA
keratin 14	LL002	IgG3	1:100	Abcam, Cambridge, UK	APC-Cy7	1:100	SBA, Birmingham, AL, USA
vimentin	V92b	IgG2B	1:50	ARA, Alphen a/d Rijn, the Netherlands	RPE	1:200	SBA, Birmingham, AL, USA
DNA					DAPI		

APC allophycocyanin
FITC fluorescein isothiocyanate
RPE R-phycoerythrin
DAPI 4',6-diamidino-2-fenylindool

Table 2 *Staining patterns*

	solid	spindle cells	stromal tissue
pankeratin	+	+	-
keratin10	+	-	-
keratin14	+	+	-
vimentin	-	+/-	+
DNA	+	+	+

For sorting, a FACSAria III was used equipped with a 50 mW 407 nm violet laser, a 20mW 488nm blue laser, a 50 mW 561 green laser and an 18 mW 635 red laser. For fluorescence collection the same filter settings were used as in the LSRII. Tumor cells were sorted using a 100 µm nozzle at 20 psi and a drop frequency of 31.2 kHz and a purity precision sorting mode (purity mask: 16, yield mask: 16). Three types of tumor subpopulations were isolated: spindle cells, tumor cells from the solid component and stromal cells, each having their own characteristic staining pattern (table 2). DNA isolation of the sorted cells was performed as described before (7) followed by DNA purification (NucleoSpin Tissue kit, Machery-Nagel, Germany).

Mutation analysis

MALDI-TOF

All samples were screened for hotspot mutations using the GynCarta 2.0 MALDI-TOF Mass Spectrometry panel (28). This panel contains the most relevant loci for gynecological malignancies on 13 different genes (*BRAF*, *CDKN2A*(p16), *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A* and *PTEN*, see Supplemental table 3 for specific mutations). Data was analyzed by two investigators separately with MassARRAY Typer Analyser software (TYPER 4.0.22, Sequenom, Hamburg,

Table 3 *Somatic mutations in solid components of VSCC*

Patient	primary	lymph node	recurrence
1	**TP53 + CDKN2A + PTEN**	**TP53 + CDKN2A**	**TP53 + CDKN2A**
2	**CDKN2A**	WT	*na*
3	**TP53 + KRAS**	*na*	WT
4	**TP53**	*na*	**TP53**
5	**TP53**	*na*	**TP53**
6	**TP53**	WT	*na*
7	**HRAS**	*na*	**CDKN2A**
8	**TP53**	*na*	*na*
9	WT	**TP53**	*na*
10	WT	WT	WT
11	**TP53 + PIK3CA + HRAS**	**HRAS**	*na*
12	**TP53° + CDKN2A**	**TP53° + CDKN2A**	*na*
13	**TP53**	*na*	*na*
14	WT	*na*	**TP53**
15	WT	*na*	WT
16	WT	*na*	WT
17	**TP53**	*na*	WT
18	**TP53 + HRAS**	WT	*na*
19	WT	WT *	**TP53 + CDKN2A**
20	WT	**TP53**	*na*
21	**CDKN2A + HRAS**	*na*	*na*
22	**TP53**	WT	*na*
23	**TP53 + CDKN2A**	*na*	*na*
24	**TP53**	*na*	*na*
25	**TP53**	**TP53**	*na*
26	WT	*na*	*na*
27	**CDKN2A**	WT	WT
28	**TP53**	*na*	**failed***
29	WT	*na*	**failed***
30	**TP53 + PIK3CA + HRAS**	WT *	*na*
31	**TP53**	*na*	WT
32	**TP53**	WT	*na*
33	**PIK3CA + PPP2R1A**	*na*	**PIK3CA**
34	**TP53 + HRAS**	**TP53 + HRAS**	*na*
35	**TP53°**	*na*	**TP53°**
36	WT	WT	*na*
37	**TP53**	*na*	WT
38	**TP53°**	*na*	**TP53° + PPP2R1A**
39	**TP53**	WT	*na*
40	WT	*na*	*na*
41	**TP53 + HRAS**	WT	*na*
42	**TP53**	*na*	**CDKN2A**
43	**CDKN2A +PTEN**	*na*	**CDKN2A**
44	**TP53**	*na*	**TP53 + CDKN2A**
45	**TP53**	WT	*na*
46	WT	WT	*na*

Table 3 Continued

Patient	primary	lymph node	recurrence
47	**TP53 + CDKN2A + HRAS**	*na*	*na*
48	**TP53**	WT	*na*
49	**TP53**	*na*	*na*
50	**TP53° + CDKN2A**	**TP53° + CDKN2A**	*na*
51	**HRAS**	**HRAS**	*na*
52	**TP53 + CDKN2A**	**TP53 + CDKN2A**	**CDKN2A**
53	WT	WT	*na*
54	**TP53**	*na*	WT
55	WT	*na*	WT
56	WT	*na*	*na*
57	**TP53°**	**TP53°**	*na*
58	WT	WT	**HRAS**
59	WT	*na*	*na*
60	WT	WT	*na*
61	WT	*na*	WT
62	WT	**TP53**	WT
63	**TP53° + CDKN2A**	**TP53° + CDKN2A**	*na*
64	WT	WT	*na*
65	**TP53 + CDKN2A**	*na*	*na*
66	**TP53**	WT	*na*

**: part of the assay failed, WT is therefore not 100% sure.*
O: a different mutation is detected on the same gene

Germany). Less than 5% of samples where scored differently by the two investigaters. These samples were analyzed by a third person working for the software provider company. This way, a consensus mutational typing was reached for all samples.

Sanger sequencing

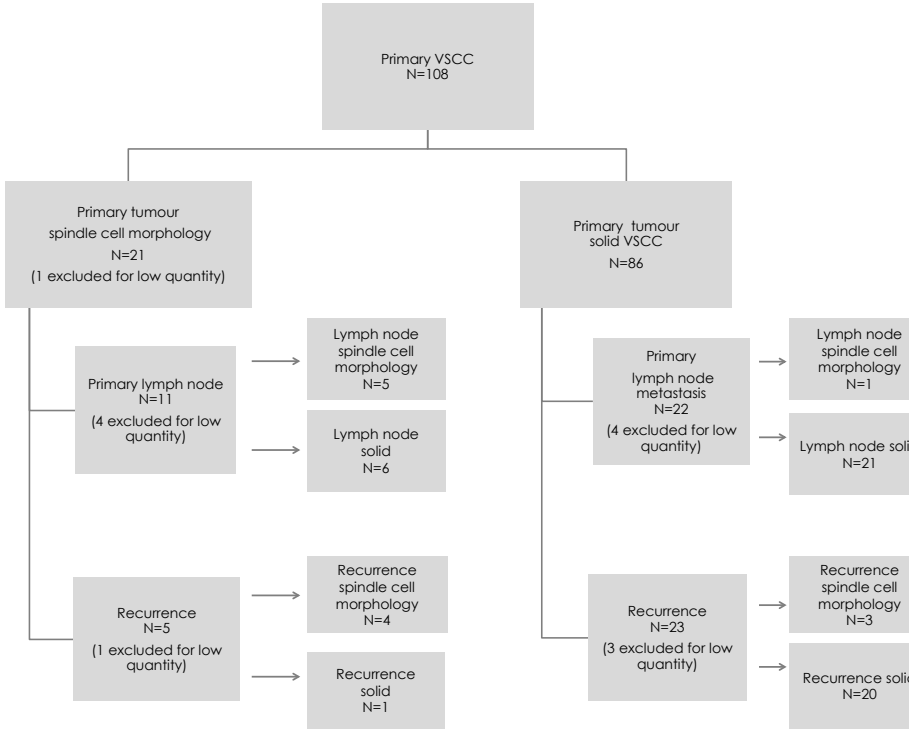
All solid tumor components were also screened for *TP53* using Sanger sequencing for exon 5-9 as described before (32). The adjecent spindle cell components did not contain enough cells to perform Sanger sequencing, so for these components only MALDI-TOF mass spectrometry was performed.

Results

Patients

Of the original cohort of 108 primary VSCCs, 22 were of the VSCC-S subtype of which one was too small for analysis and was excluded. All VSCC-S tumors were HPV negative. Of these primary VSCC-S tumors, 15 (68.2%) patients had lymph

Figure 3 *Flowchart on the included primary tumors, lymph node metastases and their adjacent spindle cell components.*



Of the original cohort of 108 patients, 22 were of the VSSC-S subtype, of which 1 was excluded for low quantity. Eleven of the VSSC-S and 22 patients of the VSSC subgroup suffered from primary lymph node metastases. 5/11 and 1/22 primary lymph node metastases contained a spindle cell component, respectively.
Five of the VSSC-S and 23 patients of the VSSC subgroup suffered from recurrent disease. 4/5 and 3/20 recurrences contained a spindle cell component, respectively.

node metastasis at time of diagnosis, whereas 26 (30.2%) of the VSCC patients had lymph node metastasis. Thirty-two patients developed recurrent disease (27.3% of VSCC-S vs 30.2% VSCC patients). Eight of the lymph nodes metastases and 4 of the recurrences contained insufficient numbers of tumor cells for our analysis and were excluded from further analysis. Six patients had spindle cell morphology in their lymph node metastasis (5 of which the primary tumor was also of spindle cell morphology), and 8 had a component of spindle cell morphology in their recurrences. In total, 66 patients were included in this study. (Figure 3)

Mutations

TP53 and *CDKN2A* were most frequently mutated in this cohort of primary tumors. The mutations detected in spindle cell morphology components, and in lymph nodes and recurrences are listed in supplemental Table 1. In lymph node metastases and recurrences *TP53* mutations occurred most frequently (10/33 lymph nodes and 8/26 recurrences), followed by *CDKN2A* (5/33 lymph nodes and 7/26 recurrences) and *HRAS* mutations(2/33 lymph nodes and 1/26 recurrences). These mutation frequencies are comparable to those in primary tumors, as described before (32). There was no difference in mutation frequency between VSCCs with and without spindle cell morphology (77 vs 58%, NS).

Recurrences

In two samples of recurrent tumour, the mutation analysis had failed. Thirty-one percent (8/26) of the recurrences showed a similar mutational profile as the primary tumor. (Figure 4 and Table 3) Another 4 (15%) recurrent tumors showed additional mutations compared to the primary tumors, while 10 (38%) recurrent tumors showed to be wild-type for a gene that was found to be mutated in the primary tumor. Four recurrent samples (15%) had simultaneously acquired a new mutation, but at the same time were wild type for a previously mutated gene, when comparing the primary and recurrent tumor.

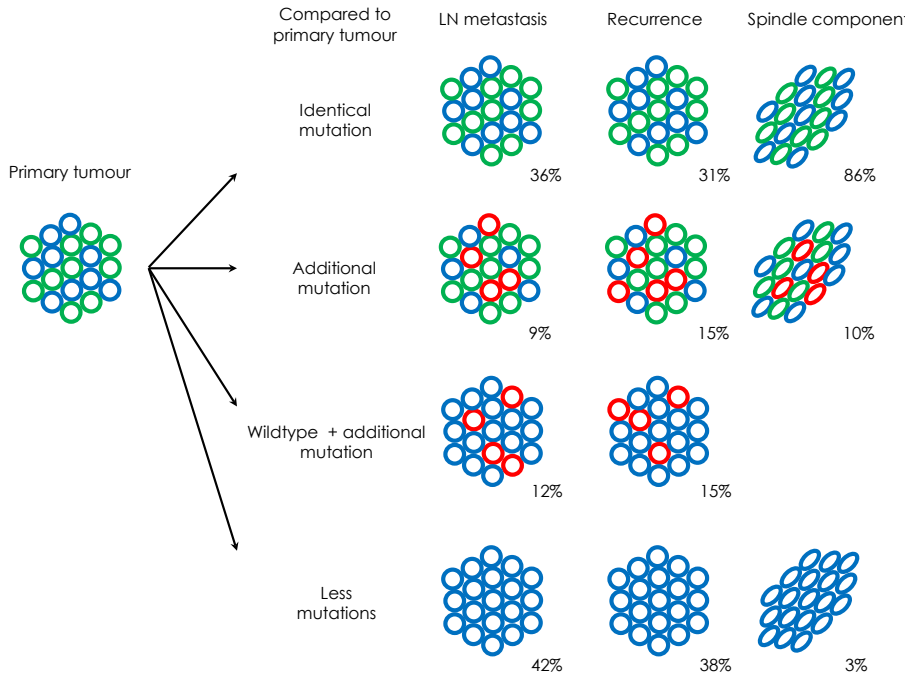
Lymph node metastases

Lymph node metastases more often had the same mutational pattern as the primary tumor, but still 21 out of 33 (64%) of the lymph node metastases had differences in mutations. In fourteen of these (42%) a mutation in the primary tumor was not detected in the lymph node, which most often was *TP53* mutation. Four patients (12%) carried a different TP53 mutation in their primary tumor than was detected in their lymph node metastases. In lymph nodes metastases from 3 cases (9%) a *TP53* mutation was detected, which was not present in the primary tumor.

Spindle cell components

Sufficient quantities of DNA suitable for somatic mutation analysis could be extracted from 83% (29/35) adjacent spindle cell components and were analyzed for somatic mutations by mass spectrometry. Spindle cell components showed an identical mutational profile as the solid component of the tumor in most cases (86%). In 4 out of 29 cases (14%) the profile differed. In 3 of these, additional mutations were detected in the spindle cell component of the tumor. In one case, the spindle cell component did not show any mutations, whereas the solid component of the tumor did harbor a *TP53* mutation and an *HRAS* mutation. There was no clear correlation between the mutational pattern of the spindle cells and that of the metastases and recurrences.

Figure 4 *Schematic representation of comparative mutational profiles*



Blue: wildtype, red and green: mutation

Figure 4 shows a schematic illustration of the differences in mutational patterns between primary tumors and spindle cell components, lymph node metastases, and recurrences. Supplemental table 3 shows the full list of detected mutations and supplemental Table 4 shows the DNA content of all FACS-sorted samples.

Discussion

We studied primary, metastatic and recurrent VSCC with spindle variants by targeted sequencing of 14 genes that are most relevant to gynaecological cancers. The results from this study show that VSCC is a disease that can harbour a large variety of mutations, with *TP53*, *CDKN2A* and *HRAS* being most frequently mutated. The spindle cell components appeared to have mutational profiles that are comparable to those of the solid component of primary tumors, so a driver mutation for spindle cell morphology could not be specified. In contrast, lymph node metastases and recurrences showed different mutational profiles compared to their solid primary tumor and spindle cell components. This might reflect the clonal selection

and / or progressive disease that acquires new mutations. Thus, somatic mutation analysis did not identify the spindle cell component as the origin of lymph node metastasis and recurrences. However, the results of this study high light that VSCCs often consist of multiple tumor clones that have different mutational spectra.

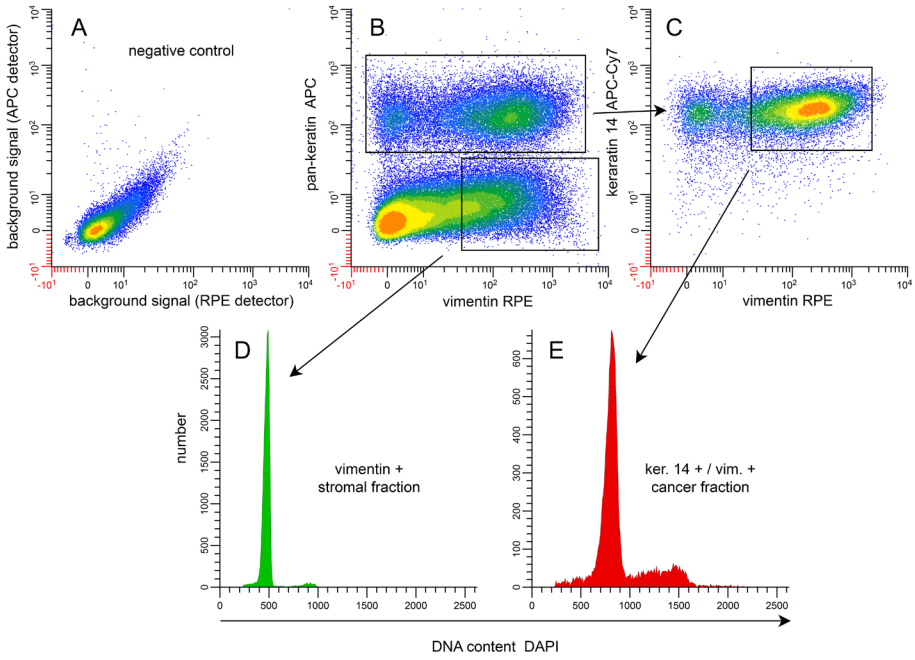
A previously published review of the literature on somatic mutations in VSCC has shown that several mutations from different pathways might be affected in VSCC (30). The mutations detected in these cohorts can be attributed to three different pathways: CDKN2A is an upstream regulator in the p53 pathway, PIK3CA, PTEN and PPP2R1A are factors in the PI3K/AKT/mTOR pathway tumor, while HRAS and KRAS are both part of the MAPK/ERK pathway. Each of these pathways is being studied as a possible target for tailored therapy, with differences in success rate (10;19;23-25;29).

In order to study molecular changes in VSCC-S subpopulations, it was first crucial to discriminate invasive tumor cells from the surrounding stromal tissue. Flow cytometry has proven to be a powerful tool in selecting different subtypes of cells (4), but its application in formalin fixed, paraffin embedded (FFPE) tissue has been limited. In a majority of studies the Hedley method was used which is based on the isolation of single nuclei from FFPE tissue blocks for DNA content analysis. However, in 2005 we published a protocol that enables the simultaneous measurement of keratin-positive carcinoma cells, vimentin-positive stromal cells as well as DNA content (5). Now we extended our protocol with two additional markers allowing a further immunophenotipically dissection of, in this case, FFPE vulvar squamous cell carcinomas into its subpopulations. (Figure 5)

A paradigm in carcinoma metastasis is that epithelial cells undergoing EMT decrease the expression of epithelial proteins such as cadherins, and gain mesenchymal traits such as vimentin expression. Spindle cells have characteristics of EMT, such as loss of cuboid shape and cell-cell junction and frequently express vimentin. Other studies have shown a relationship between upregulated vimentin expression in tumor cells and a worse prognosis (26). In VSCC too, spindle shaped tumor cells are associated with a worse prognosis (31). Here, we have successfully used these phenotypic characteristics to separate spindle cells from the solid tumor component and stromal tissue.

The low cell yield of FACS-sorted spindle cell components impaired the deployment of Sanger sequencing for TP53 analysis on these samples. Thus, *TP53* mutation analysis was limited to the solid tumor components only. In this proces of solid tumor Sanger sequencing an estimated 20% of TP53 mutations will be undetected since not all, but only the most frequently mutated exones were sequenced. Another shortcoming of this study might be the fact that targeted sequencing, as we did using mass spectometry, will always leave *un*targeted genes and mutations undetected. Future developments in mutation analysis techniques will hopefully overcome this problem in investigating low quality and low quantity tumor samples.

Figure 5 *Flow cytometry*



Flow cytometry of a lymph node metastasis containing spindle cell morphology showing separate cell populations. 4A: negative control. 4B: Cells stained for pancytokeratin and vimentin, with stromal tissue (lower population) negative for keratins and tumor tissue positive for both keratins and vimentin (upper population) 4C: tumor population, with on the right side of the cloud a tumor population expressing vimentin, a marker of epithelial-to-mesenchymal transition. 4D: diploid DNA index from stromal tissue. 4E: aneuploidy DNA index (1.65) from tumor population.

7

Several studies have shown that primary tumors often consist of multiple clones of tumor cells that, through consecutive alterations in their genome, gain distinctive variations in mutational pattern and metastatic capacity (13;21). Because we collected several tissue biopsies from different locations of the tumors, we have shown that there are many different clones of cancer cells in VSCC. The multiple mutations found in the same tumor do not necessarily reside side-by-side in one cell, but could very well originate from different clones within one tumor. As an example, case nr 53 has both a KRAS and an NRAS mutation that are typically mutually exclusive in tumors. Given the fact that metastases and recurrences often carry less mutations than the primary tumor, these results do imply that recurrences and metastases might arise from a clonal selection from the primary tumor (17). Another hypothesis could be that some of these recurrences are actually new primary tumors. The fact that we have found that nearly 30% of recurrences and nearly 10% of lymph node metas-

tases have acquired additional mutations compared to the primary tumor, might have great implications for the options for targeted therapy. When, in the future, mutations in the primary tumor are targeted by specific therapy, we have to be aware of the risk of selection of tumor cells that represent a different clone with additional, untargeted or yet untargetable mutations. It is therefore crucial to use multimodal anticancer therapies, that block multiple levels of different cancer pathways(35;36).

Although the current adagium in VSCC etiology states that vulvar cancer arises through either an HPV infection or a mutation of the *TP53* gene, our data again show that other mutations such as *CDKN2A* and *HRAS* also play a role in VSCC. The results from the present study show that lymph node metastases and recurrences from VSCC have a broad variety of mutations that are not necessarily of the same profile as the primary VSCC. VSCC-S is not associated with a specific type of mutation or mutated pathway. Also, adjacent spindle cells are likely to have the same mutational pattern as the solid component in the primary tumor. Therefore, somatic mutations are unlikely to be the causing factor for EMT or the origin of VSSC-S. Changes in the TGF-beta pathway through other mechanisms than genetic alterations could be of importance and need to be studied in more detail.

Conclusion

Mutations in *TP53*, *CDKN2A* and *HRAS* were frequently found in primary VSCC, lymph node metastases and recurrences. The results from this study show that VSCCs consist of multiple tumor clones that have different mutational spectra. There is no proof that spindle shaped cells are the origin of lymph node metastasis and recurrences. Future research on targeted therapy is needed, but we should be aware of the risk of clonal selection of cells carrying undetected or untargeted mutations.

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Supplementary tables

Supplemental table 1 *Somatic mutations in spindle cell components*

Patient	primary	spindle primary*	lymph node	spindle lymfe node*	recur-rence	spindle recur-rence*
5	WT	*na*	*na*	*na*	WT	WT
6	WT	WT	WT	*na*	*na*	*na*
8	WT	WT	*na*	*na*	*na*	*na*
9	WT	*na*	WT	WT	*na*	*na*
13	WT	WT	*na*	*na*	*na*	*na*
14	WT	*na*	*na*	*na*	WT	**CDKN2A + KRAS+ PPP2R1A**
17	WT	WT	*na*	*na*	WT	WT
24	WT	WT	*na*	*na*	*na*	*na*
25	WT	WT	WT	*na*	*na*	*na*
26	WT	WT	*na*	*na*	*na*	*na*
27	**CDKN2A**	**CDKN2A**	WT	*na*	WT	*na*
36	WT	**failed**	WT	*na*	*na*	*na*
37	WT	*na*	*na*	*na*	WT	WT
40	WT	**failed**	*na*	*na*	*na*	*na*
41	**HRAS**	WT	WT	WT	*na*	*na*
44	WT	WT	*na*	*na*	**CDKN2A**	**CDKN2A**
47	**CDKN2A + HRAS**	**CDKN2A + HRAS**	*na*	*na*	*na*	*na*
50	**CDKN2A**	**CDKN2A**	**CDKN2A**	*na*	*na*	*na*
51	**HRAS**	**HRAS**	**HRAS**	*na*	*na*	*na*
52	**CDKN2A**	**CDKN2A**	**CDKN2A**	*na*	**CDKN2A**	**failed**
53	WT	**CDKN2A + CTNNB1**	WT	**failed**	*na*	*na*
56	WT	WT	*na*	*na*	*na*	*na*
58	WT	*na*	WT	*na*	**HRAS**	**failed**
62	WT	**failed**	WT	**PTEN**	WT	WT
64	WT	*na*	WT	WT	*na*	*na*
65	**CDKN2A**	**CDKN2A**	*na*	*na*	*na*	*na*
66	WT	WT	WT	WT	*na*	*na*

Supplemental table 2 *Design for GynCarta 2.0 MALDI TOF*

GENES (13)	*BRAF*	*CDKN2A*	*CTNNB1*	*FBXW7*	*FGFR2*	*FGFR3*	*FOXL2*	*HRAS*	*KRAS*	*NRAS*	*PIK3CA*	*PTEN*	*PPP2R1A*
Mutations	p.V600E	p.R58*	p.D32A	p.R465C	p.S252W	p.R248C	p.C134W	p.G12A	p.G12A	p.G12A	p.R88Q	p.K6fs*4	**p.P179L**
	p.V600K	p.R58X	p.D32G	p.R465H	**p.P253R**	p.S249C		p.G12C	p.G12C	p.G12C	p.E542K	p.E7*	**p.P179R**
	p.V600R	p.R80*	p.D32H	p.R479Q	**p.P253L**	p.G370C		p.G12D	p.G12D	p.G12D	p.E545A	p.F37S	**p.R183G**
	p.V600L	p.D108Y	p.D32N	p.R479L	p.Y375C	p.S371C		p.G12R	p.G12F	p.G12R	p.E545G	p.R84G	**p.R183W**
		p.D108A	p.D32V	p.R505C	**p.C382R**	p.Y373C		p.G12S	p.G12R	p.G12S	**p.E545D**	p.R130*	**p.R183Q**
		p.D108C	p.D32Y		**p.N549K**	p.A391E		p.G12V	p.G12S	p.G12V	p.E545K	p.R130fs*4	**p.S256F**
		p.W110*	p.S33A		**(T>A)**	p.K650E		p.G13C	p.G12V	p.G13A	p.Q546E	p.R130G	**p.S256Y**
		p.W110X	p.S33C		p.N549K	p.K650Q		p.G13D	p.G13A	p.G13C	p.Q546K	p.R130L	**p.W257C**
		p.P114L	p.S33F		(T>G)	p.G697C		p.G13R	p.G13C	p.G13D	**p.Q546R**	p.R130P	**p.R258H**
		p.P114X	p.S33P		**p.K659E**			p.G13S	p.G13D	p.G13R	**p.Q546P**	p.R130Q	
			p.S33Y					p.G13V	p.G13R	p.G13S	**p.Q546L**	p.R173C	
			p.G34E					p.G13X	p.G13V	p.G13V	p.Y1021C	p.R173H	
			p.G34R					p.Q61H	p.Q61E	p.Q61E	p.T1025A	p.Q214*	
			p.G34V					(C>A)	p.Q61H	p.Q61K	p.T1025X	p.R233*	
			p.S37A					p.Q61H2	(T>A)	p.Q61L	p.M1043I	p.R234W	
			p.S37C					(C>G)	p.Q61H	p.Q61P	(G>A)	p.P248fs*5	
			p.S37F					p.Q61K	(T>G)	p.Q61R	p.M1043I	p.C250fs*2	
			p.S37P					p.Q61L	p.Q61K		(G>T)	p.K267fs*9	
			p.S37T					p.Q61P	p.Q61L		**p.M1043V**	p.K267fs*31	
			p.S37Y					p.Q61R	p.Q61P		p.H1047L	p.V290fs*1	
			p.T41A						p.Q61R		p.H1047R	p.L318fs*2	
			p.T41I								p.H1047Y	p.T321fs*23	
			p.T41N									p.N323fs*2	
			p.T41S									p.N323fs*21	
			p.S45C									p.R335*	
			p.S45F										
			p.S45P										
			p.S45Y										
Total (171)	**4**	**10**	**28**	**5**	**6**	**9**	**1**	**18**	**19**	**17**	**20**	**25**	**9**
Assays (99)	**2**	**5**	**12**	**4**	**5**	**8**	**1**	**8**	**7**	**6**	**13**	**22**	**6**

As published in Plos One 2014: Spaans VM, Trietsch MD, Crobach S, Stelloo E, Kremer D, Osse EM, et al. (2014) Designing a High-Throughput Somatic Mutation Profiling Panel Specifically for Gynaecological Cancers. PLoS ONE 9(3): e93451. doi:10.1371/journal.pone.0093451

Supplemental table 3 *Full list of information on detected mutations*

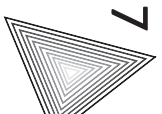
patient number	primary solid		primary spindle		lymph node solid		lymph node spindle		recurrence solid		recurrence spindle	
1	CDKN2A	p.R58*			CDKN2A	p.R58*			CDKN2A	p.R58*		
1	PTEN	p.R234W										
1	TP53	p.R273C			TP53	p.R273C			TP53	p.R273C		
2	CDKN2A	p.R80*										
3	KRAS	p.G12V										
3	TP53	p.R248W										
4	TP53	p.T150fs*16			TP53	p.T150fs*16						
5	TP53	p.C135F										
6	TP53	p.C135F										
7	HRAS	p.G12S							CDKN2A	p.R80*		
8	TP53	p.R175H										
9	no mutation detected				TP53	p.Y126fs*10						
10	no mutation detected											
11	HRAS	p.Q61R			HRAS	p.Q61R						
11	PIK3CA	p.E545K										
11	TP53	p.P250L										
12	CDKN2A	p.R80*			CDKN2A	p.R80*						
12	TP53	p.R248W			TP53	p.Y126fs*10						
13	TP53	p.G245S										
14	no mutation detected								TP53	p.R175H	CDKN2A	p.R58X
14											KRAS	p.G12S
14											PPP2R1A	p.S256Y
15	no mutation detected											
16	no mutation detected											
17	TP53	p.R175H										

Supplemental table 3 Continued

patient number	primary solid		primary spindle		lymph node solid		lymph node spindle		recurrence solid		recurrence spindle	
18	HRAS	p.G13D										
19	no mutation detected								CDKN2A	p.R80*		
19									TP53	p.R248Q		
20	no mutation detected				TP53	p.G279E						
21	CDKN2A	p.R80*										
21	HRAS	p.G13R										
21	TP53	p.P151S										
22	TP53	p.R248Q										
23	CDKN2A	p.R80*										
23	TP53	p.R273C										
24	TP53	p.R175H										
25	TP53	p.R273C			TP53	p.R273C						
25					HRAS	p.G13R						
26	TP53	p.E287D										
26	TP53	p.E294X										
27	CDKN2A	p.R80*	CDKN2A	p.R80*					CDKN2A	p.R80*		
28	PPP2R1A	p.R258H										
28	TP53	p.P152L										
28	TP53	p.R248Q										
29	no mutation detected											
30	HRAS	p.G12S										
30	PIK3CA	p.E545K										
30	TP53	p.R273C										
31	TP53	p.R249S										
32	TP53	p.R273C										

Supplemental table 3 Continued

patient number	primary solid		primary spindle		lymph node solid		lymph node spindle		recurrence solid		recurrence spindle	
33	PIK3CA	p.H1047Y							PIK3CA	p.H1047R		
33	PPP2R1A	p.R258H										
34	HRAS	p.G13S										
34	TP53	p.R273H										
35	TP53	p.D259H							TP53	p.R248W		
36	no mutation detected											
37	TP53	p.G245S										
37	TP53	p.V216M										
38	TP53	p.C176X							TP53	p.R213Q		
38									PPP2R1A	p.R183Q		
39	TP53	p.G266E										
40	no mutation detected		KRAS	p.G13S								
41	HRAS	p.G12S										
41	TP53	p.G245S										
41	TP53	p.V173G										
42	TP53	p.D186fs ins TAGC							CDKN2A	p.R80*		
43	failed for all assays											
44	TP53	p.V157F	PTEN	p.R173H					TP53	p.V157F	CDKN2A	p.R58*
44									CDKN2A	p.R58*		
45	TP53	p.P190L										
46	no mutation detected				PIK3CA	p.E542K						
47	CDKN2A	p.W110*	CDKN2A	p.W110*								
47	HRAS	p.G12S	HRAS	p.G12S								
47	TP53	p.V203L										
48	TP53	p.V173L										

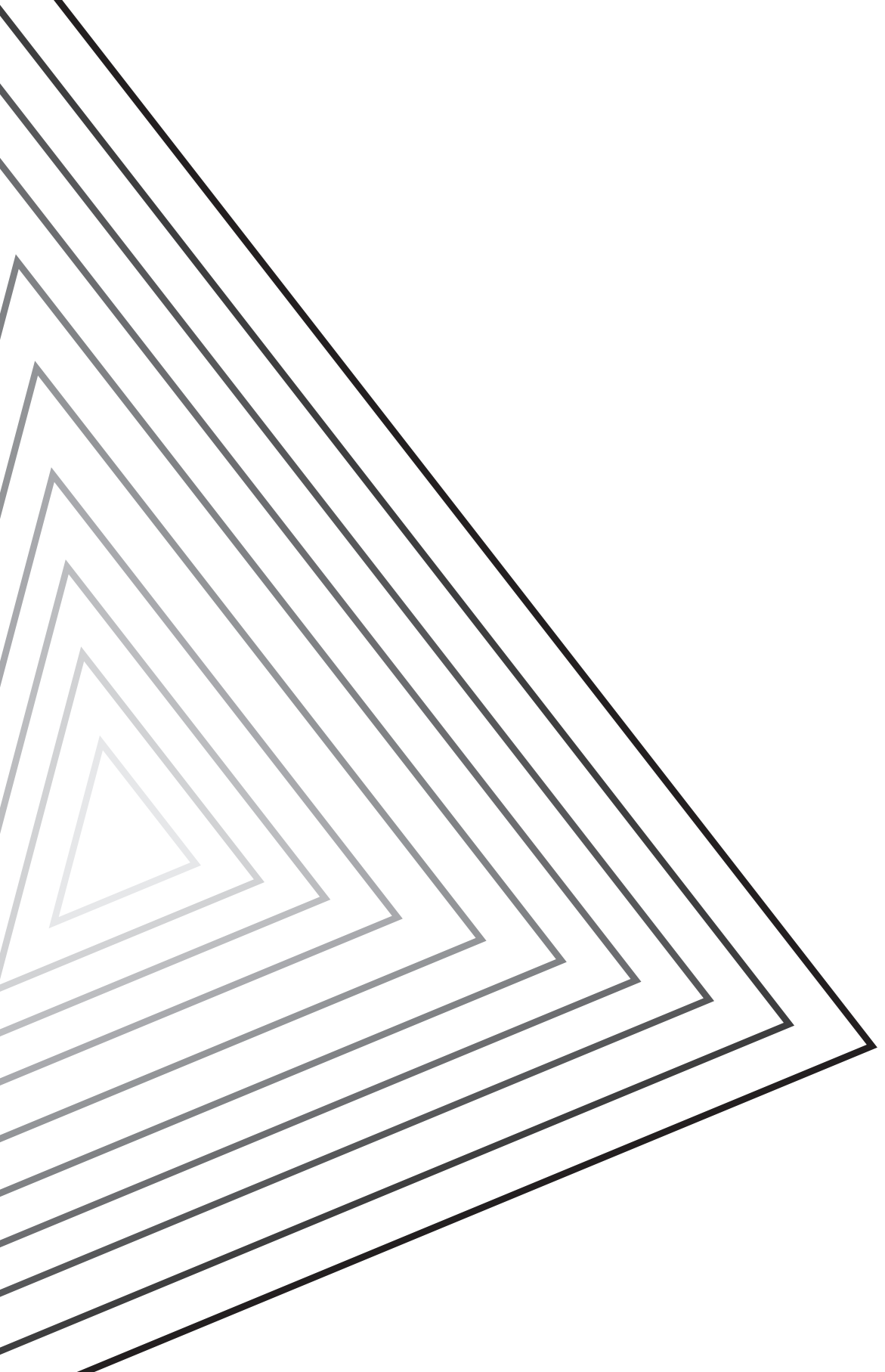


Supplemental table 3 Continued

patient number	primary solid		primary spindle		lymph node solid		lymph node spindle		recurrence solid		recurrence spindle	
49	TP53	p.R248W										
50	CDKN2A	p.R58*	CDKN2A	p.R58*	CDKN2A	p.R58*						
50	TP53	p.N267fs*3			TP53	p.R181C						
51	HRAS	p.G12D	HRAS	p.G12D	HRAS	p.G12D						
52	CDKN2A	p.R80*	CDKN2A	p.R80*	CDKN2A	p.R80*			CDKN2A	p.R80*	KRAS	p.G13S
52	TP53	p.R273C			TP53	p.R273C			PPP2R1A	p.R258H		
53	no mutation detected		CDKN2A	p.R58*			KRAS	p.G13S				
53			CTNNB1	p.S37Y			NRAS	p.G13S				
54	TP53	p.S241Y										
55	no mutation detected											
56	no mutation detected											
57	TP53	p.D148N			TP35	p.P142S						
57	TP53	p.R282W			TP53	p.R248Q						
58	no mutation detected											
59	no mutation detected											
60	no mutation detected											
61	no mutation detected											
62	TP53	p.R248Q			TP53	p.R248Q	PTEN	p.R130fs*4				
62	TP53	p.V172I			TP53	p.P142S						
63	CDKN2A	p.R58*			CDKN2A	p.R58*						
63	TP53	p.E294X			TP53	p.E294X						
64	no mutation detected											
65	CDKN2A	p.R80*	CDKN2A	p.R80*								
65	TP53	p.R273H										
66	TP53	p.C135X										

Supplemental table 4 *Copy number alterations*

Patient	primary	spindle primary	lymph node	spindle lymph node	recurrence	spindle recurrence
5					aneuploid	aneuploid
6	aneuploid	aneuploid				
8	aneuploid	aneuploid				
9			aneuploid	aneuploid		
13	aneuploid	diploid + aneuploid				
14					aneuploid	diploid
17	failed	aneuploid			aneuploid	aneuploid
24	diploid	diploid				
25	diploid + aneuploid	diploid				
26	diploid	diploid				
27	diploid	diploid				
36	diploid + aneuploid	aneuploid				
37					diploid + aneuploid	diploid + aneuploid
40	aneuploid	diploid				
41	failed	aneuploid	diploid + aneuploid	failed		
44	aneuploid	aneuploid			aneuploid	diploid + aneuploid
47	diploid + aneuploid	aneuploid				
50	aneuploid	aneuploid				
51	aneuploid	diploid				
52	aneuploid	aneuploid			diploid + aneuploid	aneuploid
53	aneuploid	aneuploid	aneuploid	aneuploid		
56	diploid + aneuppploid	aneuploid				
58					aneuploid	aneuploid
62	aneuploid	diploid + aneuploid	aneuploid	diploid + aneuploid	diploid	aneuploid
64			aneuploid	aneuploid		
65	aneuploid	diploid				
66	aneuploid	aneuploid	diploid + aneuploid	aneuploid		



Chapter 8

Summary and general discussion

Summary and general discussion

Vulvar cancer is a relatively rare gynaecologic malignancy, which accounts for approximately 3% of all cancers of the female genital tract (1). Most vulvar cancers are squamous cell carcinomas (VSCC), a cancer arising from epithelial cells. Currently, it is commonly accepted that VSCC can arise through two separate pathways (2;3). The first is strongly associated with a persistent infection with high risk human papilloma virus (hrHPV), which affects younger patients than the second type of VSCC does. The second, HPV independent type of VSCC is associated with lichen sclerosus and mutations in the *TP53* gene. The aetiology of HPV positive vulvar cancer is quite well understood, but the HPV independent axis remains to be unravelled. This thesis aims to gain knowledge on the origin of this type of vulvar cancer. Using genetic and morphologic profiling, we investigated prognostic and etiologic factors in HPV positive and negative vulvar cancers.

Technical developments in genetics are rapidly improving. While many years ago, it would take weeks to investigate a single exon of a single gene, we are now able to study the full genome of a human being within days. These tests generate massive amounts of data, much of which we do not know its value yet. For the time being, it is therefore crucial to think ahead before spending lots of money, time and tissue on 'random' genetic testing. Instead, it might be more useful to make a preselection of genes that are likely to be relevant for the specific type of cancer and the specific research question. **Chapter 2** describes the design of a somatic mutations profiling panel using mass spectrometry that is created especially for gynaecological cancers, focussing on the mutations that are most relevant or prevalent in cervical, endometrial, ovarian, and vulvar cancer. Mass spectrometry is a reliable technique that requires low quantities and quality of DNA, which is perfect for the scarce VSCC samples that are usually formalin fixed and paraffin embedded.

A review of the literature and frequency data from online mutation databases pointed out that for vulvar cancer, only a few genes have been studied (4). *TP53* is of course a major player, but mutations in *CDKN2A* and *PTEN* have been described too. *HRAS*, *KRAS* and *NRAS* have also been studied, but no mutations have been detected in these genes. Because the limited amount of research on somatic mutations in vulvar cancer, we had to rely on mutational frequencies from other gynaecological cancers, and from cancer types that have similarities with vulvar cancer, such as squamous cell carcinoma of the skin and of the head and neck. Using a training set of 51 samples from different types of gynaecological cancers, we were able to adjust and improve the panel technically. Also, recent literature on newly discovered somatic mutations could be used to add and replace new assays. The final 'GynCarta' panel consisted of *BRAF*, *CDKN2A*, *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, and *PTEN*. *TP53* is miss-

ing from this list, because mutations occur over the full length of the gene instead of on hot spots and is therefore not suitable for this technique. The panel has been used to test and was validated in a total of 546 tumours, of which 25 were VSCCs. Even with very low DNA quality, we were able to detect small amounts of mutated alleles. To assess assay reproducibility, we tested a percentage of the samples in duplicate and in triplicate, resulting in a reproducibility of more than 95%. In the first 25 VSCC samples that we used to test and validate the panel, *CDKN2A*, *PIK-3CA* and *HRAS* were found to be mutated, of which the last two mutations had not been described in vulvar cancer before. Unexpectedly, we did not detect any *PTEN* mutations in the 25 VSCC samples. In conclusion, the newly designed 'GynCarta' panel had shown to be functional and reproducible, but still has its limitations. Only hot spot mutated genes that are preselected can be studied, so non-hot spot genes such as *TP53* or *ARID1A* cannot be investigated using this method. As other techniques such as whole genome sequencing and Next Generation Sequencing are rapidly improving in terms of processing time and cost, we expect that mass spectrometry can be used for now, but will in the end be replaced by techniques that give more information, maybe even more than needed.

Chapter 3 contains the results from using the GynCarta panel to study a cohort of 108 vulvar cancer patients. The GynCarta panel was supplemented by Sanger sequencing of *TP53*, because of its known role in the pathogenesis of HPV negative VSCC. Samples were collected from the Leiden University Medical Center pathology archives from vulvar cancer patients that have been surgically treated between 2000 and 2009. Follow up data until December 2012 were collected from medical records and from the institutes oncologic database, thus creating a large cohort of VSCC patients that was used throughout this thesis. For the mutational analysis, a sufficient quantity and quality of DNA had to be extracted from the samples. One of the tumours was of such low quality that it could not be used for further mutational analysis. Of the remaining 107 VSCCs, 66 tumours (62%) contained at least one mutation. Mutations were detected in 7 different genes: *TP53* was mutated in respectively 58 of the cases, *CDKN2A(p16)* in 14, *HRAS* in 10, *PIK-3CA* in 7, *PPP2R1A* in 3, *KRAS* in 1 and *PTEN* in 1 case. Surprisingly, we did not find as many *PTEN* mutations as expected based on mutation frequencies that have been reported before by Holway et al (5), a frequently cited paper on somatic mutations in vulvar cancer. Knowing that our technique can only find hot spot mutations that have been selected for the GynCarta panel, we are aware of the fact that some *PTEN* mutations may have stayed undetected. We therefore also stained full FFPE section using a PTEN antibody, but still we found no more than 5% of the tumours to have lost PTEN expression. PTEN can be downregulated by mutations in the *PTEN* gene, but also by epigenetic mechanisms, so the 5% aberrant staining might only partially be caused by somatic mutations that have been undetected by the GynCarta panel. Therefore, the possibility exists that the frequency of *PTEN* mutations

has been overestimated by Holway et al., possibly because of an unknown selection bias or by pure chance.

All samples in our cohort have been screened for presence of HPV. Seventeen percent of them tested positive for high-risk HPV. Mutations were most frequently found in HPV negative samples, but in three cases of HPV positive samples, a mutation in *TP53* was also found. These results confirm the hypothesis that HPV infection and *TP53* mutations play a key and almost separate role in the pathogenesis of vulvar cancer, but they also show that TP53 is not alone. HPV can inactivate *p53* by inducing its degradation by E6 protein (6), so, even though the two pathways have very separate starting points, there are also many similarities in cell cycle disruption and oncogenesis (3;7). Our data showed that the five-year survival was significantly worse for patients with a somatic mutation (47% vs 59%, p=0.035). When corrected for possible confounders age, tumour size and HPV infection, an *HRAS* mutation is an independent prognostic marker with a hazard ratio of 2.848 (95% CI 1.113–7.290). *TP53* mutations and HPV infection were no independent prognostic markers. *TP53* mutations are the mutations that are found most often in vulvar cancer and are thought to be early events in the genesis of vulvar cancer because it is also found in dVIN and lichen sclerosis lesions (8-17). Possibly, additional mutations such as *CDKN2A* and *HRAS* represent the cascade of tumour instability and progression. Our results are of great importance, since much research is being performed on the potential targeting of the downstream pathway of *RAS* mutations (18-20).

The data from chapter 3 were compared to the previously published studies in **chapter 4**. A review of the current literature gives an overview of what is currently known of genetic and epigenetic alterations in vulvar cancer and its precursor lesions. Genetic alterations, such as somatic mutations, but also allelic imbalances, microsatellite instability or copy number alterations have been studied. Epigenetic alterations are defined as heritable changes in the gene expression without changes in the DNA sequence. For vulvar cancer, 9 papers were found on epigenetic alterations, all focussing on hypermethylation. Hypermethylation frequencies varied greatly between LS, VIN and VSCC. Much more research has been done on genetic alterations, as 59 publications were included in our review, of which 34 focussed on somatic mutations, 24 on copy number changes and allelic imbalances and 3 on microsatellite instability. Somatic mutations and copy number alterations were observed more frequently in higher stages of both precancerous and cancerous lesions. HPV negative vulvar cancers harboured more mutations than HPV positive tumours, which is in line with the results we presented in chapter 3. These findings confirm the two-pathway hypothesis in which vulvar cancer arises either through an HPV infection or through lichen sclerosus and/or a mutation in *TP53*. *TP53* was studied most, and therefore alterations in this gene make a substantial portion of the reported mutations in our review of the literature. We do have to be aware that these results might be a 'self-fulfilling prophecy': if *TP53* is the only gene you

are looking at, it will be the only gene that you find to be mutated. Decades ago, when the first studies on genetic alterations in vulvar cancer were performed, the methods to detect mutations were limited and studying just one single gene could take a very long time. Because of this, the 'low hanging fruit', i.e. the genes that are likely to be mutated based on research in other types of cancer, have been picked first. More recent studies, including the papers we described in chapter 3, show that several other genes are frequently mutated in vulvar cancer. Future research using Next Generation Sequencing or other techniques that can process the whole genome, might find many more mutations of which we do not know its existence and importance yet.

The majority of vulvar cancers are squamous cell carcinomas, but within this cancer type, morphological sub classifications can be made with a possibly different clinical behaviour and prognosis. Understanding more of the differences in behaviour of various carcinomas may help us to predict the risk of recurrence or progression and may influence treatment strategies. **Chapter 5** describes a vulvar squamous cell carcinoma type called carcinoma with spindle cell morphology. Spindle cell carcinoma, sometimes referred to as pseudosarcoma or carcinosarcoma, has been described in many different types of cancer and is associated with a worse prognosis in head-and-neck squamous cell carcinoma and in oesophageal and lung cancer (21-25). Spindle shaped epithelial cancer cells have lost their epithelial characteristics and seem to gain the ability to infiltrate the underlying stroma more easily (26). True spindle cell carcinomas are quite rare and are estimated to occur in 1-5% of VSCC cases. More often, a mixed morphology is seen in vulvar squamous cell carcinomas: a conventional, solid component of the tumour with an adjacent component of spindle cell morphology. We hypothesise that this type of VSCC is related to spindle cell carcinoma and shares some of its aggressive potential. In this chapter, we suggest criteria for VSCC with spindle cell morphology and we describe a cohort of 108 consecutive VSCC patients, of which 20% turned out to have a component with spindle cell morphology, all of these being HPV negative. These patients had a worse prognosis when compared to conventional, solid VSCC. Overall survival differed significantly between patients with and without spindle cell morphology, with a 5-year overall survival of 27.3% vs 58.2% (*P*=0.00041), respectively. Disease-specific survival was significantly worse for patients with spindle cell morphology compared to patients with non-spindle cell morphology VSCC, with a 5-year disease-specific survival respectively 45.2% and 79.7% (*P*=0.00057). When correcting for potential confounders such as age, HPV infection and tumour characteristics, spindle cell morphology was an independent prognostic factor for poor survival, with a hazard ratio of up to 4.1 (95%CI 1.61–10.60). Despite the fact that our cohort was relatively large in the context of a rare disease such as vulvar cancer, our findings need to be confirmed in larger groups of VSCC patients before we can truly interpret its clinical value.

To further investigate risk factors and morphologic predictors of a poor prognosis we investigated L1CAM expression in vulvar cancer. **Chapter 6** reports the prevalence and prognostic value of L1CAM expression in two groups of patients with vulvar cancer from two different university medical centres in the Netherlands. L1CAM is a cell adhesion molecule that plays a crucial role in in the development of nerve tissue, but is also expressed by certain types of tumours (27-29). In vitro studies have shown that L1CAM can also function as a promoter of cell motility (30). In this chapter, we have stained two sets of 103 and 245 vulvar cancer samples, respectively, for L1CAM expression and correlated it to survival. We found that L1CAM expression was related to a poor prognosis. Five-year survival was worse for patients with L1CAM expression (overall survival 46.1% vs 63.6%, P=.014, as was disease specific survival (63.8% vs 80.0%, P=.018). Multivariate analysis indicated L1CAM expression as an independent prognostic marker (HR 2.9, 95% CI 1.10–7.68). We found that L1CAM was most often expressed at the invasive border of the tumour or by spindle shaped tumour cells. To understand more about the role of L1CAM expression on prognosis, we performed an in vitro spheroid invasion assay, which showed decreased invasion of L1CAM-expressing VSCC spindle cells after treatment with L1CAM-neutralising antibodies. Currently, there are three hypotheses on how L1CAM is upregulated in cancer. We investigated which of these hypotheses might apply to our cohort of vulvar cancers. First, in colorectal cancer, β-catenin (*CTNNB1*) mutations result in upregulation of L1CAM (31). None of our samples contained any β-catenin mutations, so this is not likely to be the driving cause for L1CAM expression in VSCC. Secondly, p53 has been suggested to be a regulator of L1CAM expression (32). In our cohort, there was no relation between*TP53* mutations and L1CAM expression, so this also does not seem to be a likely explanation in our study cohort. This leaves us with the third and last hypothesis, which states that L1CAM is upregulated in the process of Epithelial to Mesenchymal Transition (EMT) (33;34). During EMT, epithelial markers such as e-cadherin are lost, whilst mesenchymal markers such as vimentin are gained. These changes dissolve the cell-cell adhesions, giving cancer cells the opportunity to bud out of the epithelial matrix. Hereby, they can infiltrate underlying tissue and metastasise further into the body. Spindle cell carcinoma and cancers with spindle cell morphology have an invasive and potentially metastatic growth. This type of cancer progression is in some cases also thought to develop through the process of EMT (35). Our findings show that L1CAM was exclusively expressed at the invasive border of the tumour in areas with EMT-like growth patterns, suggesting an association between EMT and L1CAM. In vitro studies in our laboratory confirmed this hypothesis by showing that spindle shaped vulvar cancer cells highly express L1CAM protein, whereas their cobble shaped counterparts express L1CAM in very limited amounts, or do not express L1CAM at all.

In **Chapter 7**, the relation between mutational and morphological data was further analysed. All primary vulvar cancer samples in our cohort had been screened for somatic mutations in Chapter 3 and for spindle cell morphology in Chapter 5, and we expanded this to lymph node metastases and recurrences. We have already shown that VSCC with spindle cell morphology is more likely to be aggressive than the non-spindle cell morphology VSCCs. We hypothesized that the spindle shaped tumour cells are the precursors of metastasis and recurrences. If spindle cells have a distinct mutational profile from solid tumour components, this could provide us with a label that could help recognise which part of the primary tumour is the precursor of lymph node metastasis and recurrences. We have designed a new flow cytometry technique to separate spindle cells from the surrounding tissue. This way, we could extract DNA purely from spindle cells, instead of mixing it with other, non-spindle, tumour cells or stromal cells. Allelic imbalances occurred in both the spindle and the non-spindle cells. Studying somatic mutations also did not provide a distinct profile for spindle shaped tumour cells, but it did show that recurrent and metastatic tumours were likely to differ from their primary tumour. We were unable to prove our hypothesis of a selective role for somatic mutation in the onset of spindle cell morphology, nor for its progression to metastases and recurrences, but did show that vulvar cancers probably consist of multiple clones with multiple mutational spectra. This has implications for further research on targeted therapy, since one clone might be targeted, while the others might remain unaffected by the therapy.

Glance into the future of vulvar cancer research:

The data gathered in this thesis has provided us with novel insight in the pathogenesis of vulvar squamous cell carcinoma. As described before by van der Avoort et al. (3) we have also shown that HPV related VSCC and VSCC induced by somatic mutations are two very different diseases (3;7). The key role of mutations in *TP53* has been confirmed in numerous studies, but this thesis stresses that there are multiple other mutations that might contribute to vulvar carcinogenesis. Technical developments in basic oncological research are improving fast. The use of Next Generation Sequencing will soon be affordable and fast enough to thoroughly investigate VSCC and its precursor lesions. Finding new somatic mutations in vulvar cancer will help us further understand its sometimes aggressive behaviour, and could help us discriminate high risk from low risk tumours.

Surgical excision will probably remain the corner stone of vulvar cancer treatment, but targeted chemo-, antibody- or biological response modifier treatment could be of clinical value for patients with inoperable tumours and precursor lesions. A possible example is the recent work by Kiessling et al. (36) which shows that MEK and mTOR inhibitors reduce cell growth in *HRAS* mutated cancer cell lines, a

gene we found to be mutated in nearly 10% of vulvar squamous cell carcinomas (36). We must carefully choose combinations of treatment modalities, because targeting only one alteration might induce clonal selection and resistance (37;38).

As shown in our work on L1CAM expression, some of the steps of the cascade of cancer development from vulvar intraepithelial neoplasia towards invasive vulvar cancer might be tackled by targeted therapy. We have developed several vulvar cancer cell lines, with and without spindle cell morphology, and with different expression levels of L1CAM. Our pilot *in vitro* study has demonstrated that blocking L1CAM inhibits tumour growth, as described in chapter 6. We are currently developing a novel orthotopic vulvar cancer mouse model to study the effect of L1CAM inhibition *in vivo*. The first results are very promising and confirm our hypothesis that blocking L1CAM inhibits tumour growth.

Another possible targetable factor is the Transforming Growth Factor-β pathway. TGF-β is a major inducer of EMT, which could be an important inducer of spindle cell morphology in vulvar cancer. TGF-β family members influence proliferation, differentiation and apoptosis in healthy epithelial tissue and should have a tumour suppressive role. In advanced stage tumours, however, TGF-β seems to gain a tumour promoting role, where it stimulates cell motility and angiogenesis (39;40). One of the modulators of TGF-β signalling is Endoglin. This co-receptor is highly expressed by proliferating endothelial cells and is crucial in angiogenesis (41-43). Endoglin is therefore found in high amounts on tumour vasculature, but is also expressed by some other tumour cell types, such as in prostate cancer, where it is thought to inhibit the metastatic and migratory capacity of cancer cells (44;45). Surprisingly, we found strong expression of endoglin on spindle shaped vulvar cancer cells, where it seems to have an opposite association: infiltrating spindle shaped tumour cells express endoglin in higher amounts than solid, coble shaped and non-infiltrating tumour cells do. Endoglin inhibition in advanced cancer patients has been studied in a phase 1 trial, using the anti-endoglin-antibody TRC105 (46). *In vitro* studies that we have performed on vulvar cancer cell lines showed that neutralising endoglin with TRC105 markedly decreases the invasion of tumour cells. Interestingly, this effect was only seen in spindle cell morphology, and not in L1CAM negative cobble shaped tumour cells. This might be a new path to explore, which will hopefully lead to new treatment strategies of the more aggressive subtypes of vulvar cancer. We do hope that our data will contribute to a new chapter in vulvar cancer research, which will open doors to new possibilities in understanding and treating vulvar cancer patients.

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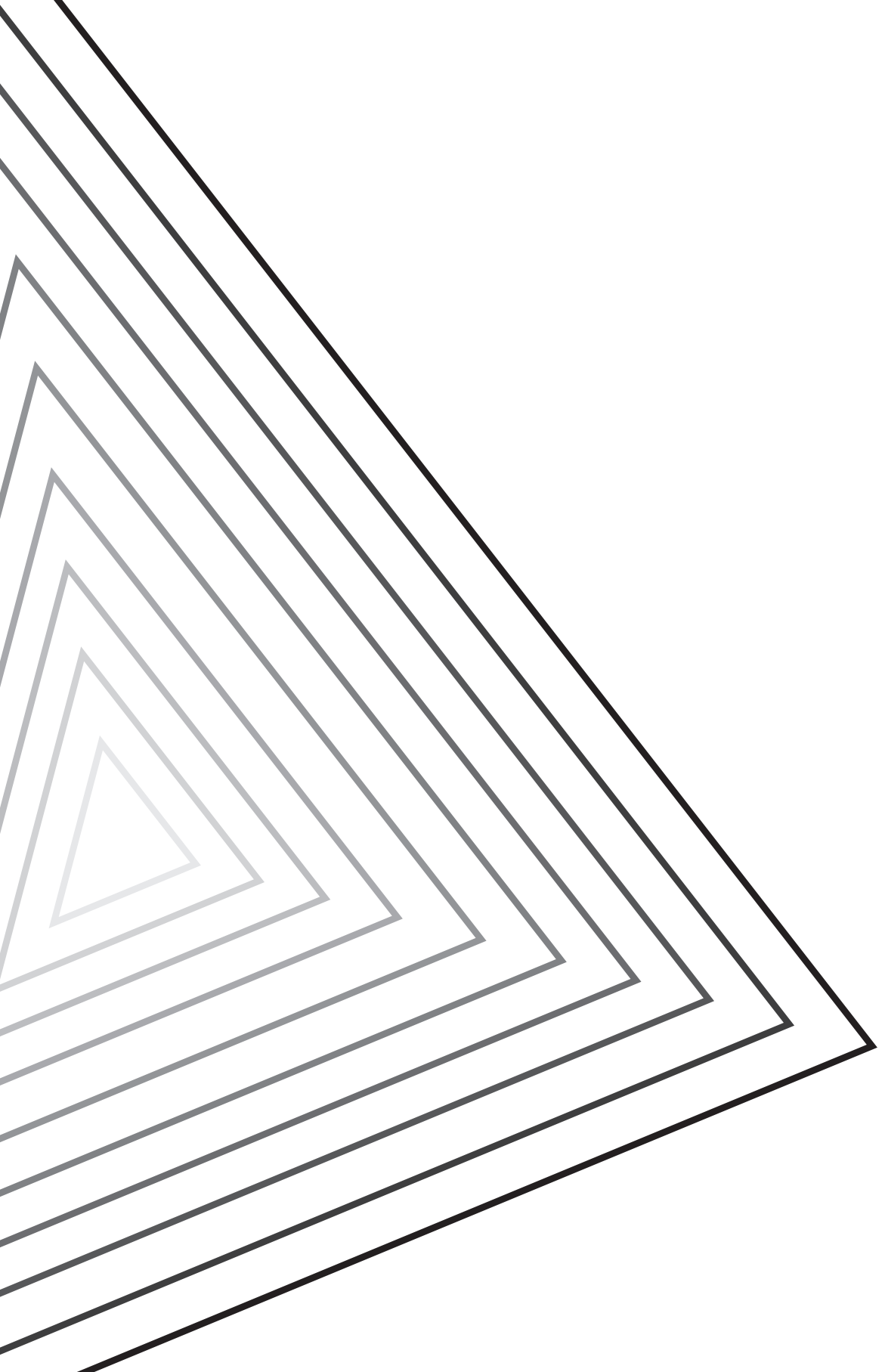

Addendum

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Nederlandse samenvatting

Inleiding

De vulva is het uitwendige deel van het vrouwelijk geslachtsorgaan en bestaat uit de kleine schaamlippen, rond de opening van de vagina, de uitgang van de urinebuis en de clitoris. Vulvacarcinoom is een kwaadaardige aandoening van de huid van de vulva die gelukkig weinig voorkomt, maar wel een grote invloed heeft op het leven van de vrouwen die het treft. In Nederland wordt ieder jaar bij 2 op de 100.000 vrouwen vulvacarcinoom vastgesteld. Als een vulvacarcinoom in een vroeg stadium wordt ontdekt, is curatieve behandeling goed mogelijk en heeft de patiënte een goede prognose. De standaardbehandeling bestaat uit chirurgische excisie van de tumor en het omliggende weefsel (radicale excisie) en in veel gevallen het verwijderen van de lymfeklieren in de lies. Sommige vrouwen krijgen echter snel een recidief of hebben een tumor die snel en progressief groeit. Wanneer de tumor zich heeft uitgebreid tot de urinebuis, de anus of de lymfeklieren zal de tumor ruimer moeten worden uitgesneden om een veilige tumorvrije marge te kunnen bereiken. Dit resulteert helaas ook in schade aan gezonde weefsels. Deze soms verminkende behandeling heeft om die reden een grote impact op de kwaliteit van leven van patiëntes, omdat zij last kunnen hebben van wondgenezingsstoornissen, lymfoedeem en zenuwschade. Dit kan weer leiden tot seksuele problemen en incontinentie.

De ontstaanswijze van vulvacarcinoom wordt op dit moment nog niet goed begrepen. Er bestaan verschillende vormen van schaamlipkanker, en de meest voorkomende is het plaveiselcelcarcinoom. Inmiddels staat vast dat ongeveer 40% van deze plaveiselcelcarcinomen is geassocieerd met een persisterende infectie met een hoog-risico variant van het Humaan Papilloma Virus (HPV). De resterende 60% draagt geen HPV en is geassocieerd met mutaties in het gen *tp53*.

Op dit moment is het wel of niet hebben van lymfekliermetastasen de enige goed bruikbare maat waarmee voorspeld kan worden of iemand een goede of slechte prognose heeft. In dit proefschrift proberen we beter te begrijpen hoe vulvacarcinoom ontstaat en zoeken we naar een methode om te voorspellen welke vulvacarcinomen zich agressief zullen gedragen.

Behandeling van vulvacarcinoom

De Internationale Federatie voor Gynaecologie en Obstetrie (FIGO) heeft een stadieringsysteem ontwikkeld waarmee patiënten kunnen worden ingedeeld in risicocategorieën op basis van weefselkenmerken en klinische parameters. Patienten met een tumor in het laagste stadium hebben een vijfjaarsoverleving van 79%, terwijl patiënten in het hoogste stadium een vijfjaarsoverleving van slechts 13% hebben.

De behandeling van eerste keus voor het laagste stadium vulvacarcinoom is chirurgische excisie. Afhankelijk van de grootte en de doorgroei van de tumor komen patiënten in een hoger FIGO stadium en zal de behandeling ook agressiever zijn, waarbij de lymfeklieren kunnen worden verwijderd, of bestraling en chemotherapie kan worden gegeven.

Ontstaan van vulvacarcinoom: twee verschillende routes:

Wanneer cellen ongecontroleerd gaan delen ontstaat kanker. Dit kan gebeuren wanneer er in het DNA in de cel een fout ontstaat die niet hersteld wordt. In principe kan dit in elk type cel in het menselijk lichaam gebeuren. Huidkanker is wereldwijd een van de meest voorkomende vormen van kanker. In de meeste gevallen ontstaat huidkanker nadat er DNA-schade is veroorzaakt door ultraviolette straling uit zonlicht. Veelvoorkomende types huidkanker zijn onder andere melanoom, basaalcelcarcinoom en plaveiselcelcarcinoom.

Ook in de huid van de vulva kan kanker ontstaan. Dit gebeurt echter niet door zonlicht zoals in de huid op de rest van het lichaam, maar door een compleet ander mechanisme. Zoals eerder genoemd, zijn de meeste gevallen van vulvacarcinoom plaveiselcelcarcinomen. Plaveiselcelcarcinomen ontstaan vanuit het plaveiselepitheel van de huid.

Ongeveer 40% van alle vulvacarcinomen ontstaat na een langdurige infectie met hoog risico HPV. HPV's zijn veelvoorkomende en besmettelijke virussen die abnormale celgroei kunnen veroorzaken. Hierdoor kunnen bijvoorbeeld wratjes ontstaan, maar in sommige gevallen ook kanker. Meer dan 100 verschillende varianten van HPV zijn al beschreven. Ongeveer 15 hiervan zijn 'hoog risico' varianten die kanker kunnen veroorzaken. Deze hoog risico HPV's staan vooral bekend als veroorzaker van baarmoederhalskanker en voorlopers daarvan. Veel minder vaak veroorzaken deze virussen ook vulvacarcinomen. Geschat wordt dat ongeveer 80% van alle vrouwen tenminste eens in haar leven een HPV-infectie doormaakt. Slechts een heel klein deel van deze vrouwen zal HPV-gerelateerde kanker ontwikkelen. In de meeste gevallen is de HPV-infectie voorbijgaand van aard en ruimt het lichaam het virus binnen 1 tot 2 jaar op. Sommige vrouwen blijken echter niet in staat dit virus te klaren, bijvoorbeeld als het immuunsysteem niet goed functioneert. Het virus kan zich dan in het DNA van de aangedane cellen nestelen. De cellen zullen hierdoor ongecontroleerd gaan groeien en kunnen uiteindelijk uitmonden in kanker. Vrouwen die roken en vrouwen die een verminderd immuunsysteem hebben door bijvoorbeeld HIV/AIDS hebben vaker een persisterende HPV-infectie en dus ook vaker HPV-geassocieerde vormen van kanker. We begrijpen nog niet goed waarom het sommige vrouwen zónder deze risicofactoren ook niet in staat zijn het virus te klaren. HPV-positieve vulvacarcinomen worden voorafgegaan door een premaligne huidaandoening 'usual vulvar intraepithelial neoplasia', afgekort uVIN. Vrij vertaald staat dit voor 'meest voorkomende nieuwvorming in het epitheel van de huid van

de vulva', met als kenmerk dat er wel afwijkende cellen in de epitheellaag van de huid aanwezig zijn, maar dat deze niet doorgroeien door de basaalmembraan die de huid van het onderliggende weefsel scheidt. De voorlopers van baarmoederhalskanker die door HPV worden veroorzaakt gaan meestal onopgemerkt, terwijl uVIN juist vaak klachten van jeuk en pijn kan geven. Onbehandelde patiënten met uVIN ontwikkelen in 9-16% van de gevallen een maligniteit die wél doorgroeit. Bij patiënten die wel behandeling krijgen is dit nog 3%. Dit HPV-positieve type vulvacarcinoom komt vaker voor bij jonge vrouwen met een gemiddelde leeftijd van 40 jaar. Het is geassocieerd met roken en promiscuïteit.

De overige 60% van de vulvacarcinomen is niet geassocieerd met HPV en komt juist vaker voor bij oudere vrouwen, met een gemiddelde leeftijd van 70 jaar. Dit type kan worden voorafgegaan door lichen sclerosus, een chronische autoimmuunontsteking van de huid. In sommige gevallen kan de voorloper 'differentiated vulvar intraepithelial neoplasia' (dVIN) worden gevonden. Gedifferentieerd betekent in dit geval dat de tumorcellen ver zijn uitgerijpt en sterk lijken op het oorspronkelijke weefsel. Dit type VIN is dan ook zeer moeilijk te herkennen, en wordt vaak pas gezien als er al vulvacarcinoom is ontstaan. Drie tot 5% van alle vrouwen met lichen sclerosis ontwikkelt uiteindelijk kanker. Deze groep vrouwen met vulvacarcinoom hebben een slechtere prognose dan de vrouwen met een HPV-geassocieerde tumor.

Genetische mutaties in HPV negatieve vulvacarcinomen

De HPV-negatieve vulvacarcinomen worden geassocieerd met lichen sclerosis, maar ook met genetische mutaties. Het meest bestudeerde gen waarin ook de meeste mutaties zijn gevonden is het gen *tp53*. *Tp53* is de bewaker van de celcyclus: wanneer er een fout wordt ontdekt in het DNA van een cel, zal *tp53* de celdeling afremmen. Wanneer *tp53* beschadigd is geraakt door een mutatie kan de cel ongecontroleerd gaan delen en ontstaat er instabiliteit in de chromosomen. Er is bij vulvacarcinomen weinig onderzoek gedaan naar mutaties in andere genen dan in *tp53*. In kleine studies hebben Holway en Growden laten zien dat een deel van de vulvacarcinomen mutaties in het gen *PTEN* dragen. O'Nions, soufir en Gasco hebben mutaties in *CDKN2A* aangetoond. *PTEN* en *CDKN2A* zijn allebei tumorsuppressorgenen die onderdeel uitmaken van de signaalstructuur die de celcyclus kunnen afremmen en cellen zelfs tot apoptose kunnen doen overgaan. In melanomen, long-, colorectaal- en borstkanker is al aangetoond dat tumorspecifieke mutaties gebruikt kunnen worden als voorspeller voor overleving, en zelfs voor tumorspecifieke behandelstrategieën. Een aantal studies naar dit soort gerichte therapieën hebben veelbelovende resultaten laten zien, zoals bijvoorbeeld in de PI3K/AKT/mTOR route bij colorectaalkanker.

Epigenetische veranderingen

Een genetische mutatie houdt in dat een deel van de DNA volgorde in een cel veranderd is. Vulvacarcinomen ontstaan mogelijk ook ten gevolge van een ander soort verandering, namelijk 'epigenetische' verandering. Epigenetische veranderingen zoals hypermethylatie zijn overerfbare veranderingen in genexpressie, zonder dat er een verandering in de DNA volgorde optreedt. Hypermethylatie werkt als een schakelaar die genen aan en uit kan zetten. Hiermee kan bijvoorbeeld een tumorsuppressorgen uitgeschakeld worden. In vulvacarcinomen is hypermethylatie van onder andere *RASSF2A*, *MGMT* en *TSP1* beschreven.

Morfologische factoren met voorspellende waarde

Zoals eerder genoemd gebruikt de FIGO stadiering klinische en histologische parameters. Deze stadiering heeft zijn voorspellende waarde bewezen in grote groepen patiënten. Om de mortaliteit en morbiditeit bij patiënten met vulvacarcinoom nog lager te kunnen krijgen is het belangrijk onderzoek te blijven doen naar nieuwe, aanvullende risicofactoren. Op dit moment zijn lymfekliermetastasen de enige betrouwbare maat voor overleving en de kans op een recidief. In dit proefschrift beschrijven we morfologische karakteristieken die mogelijk kunnen bijdragen aan het maken van een juiste voorspelling. Vulvacarcinomen met een sprieterig groeipatroon hebben mogelijk een slechtere prognose dan 'gewone', in solide veldjes groeiende vulvacarcinomen. Deze sprieterige spoelvormige cellen, of spindle shaped cells in het engels, zijn dunner en groeien niet in eilandjes, maar als losse langgerekte cellen of in sprieterige uitlopers. Spoelcellige tumoren komen voor bij veel soorten tumoren en worden bij slokdarm-, long-, mond- en keelkanker geassocieerd met een slechtere prognose. De spoelvormige cellen lijken hun epitheliale karakter te verliezen en hebben een groter vermogen zich los te maken van de oorspronkelijke tumor en in het onderliggende weefsel te infiltreren.

Epitheliale naar Mesenchymale Transitie

Onderzoek wijst uit dat invasief groeiende vulvacarcinomen wellicht ten dele Epitheliale naar Mesenchymale Transitie (EMT) ondergaan. EMT is een proces dat tijdens de embryonale ontwikkeling plaatsvindt, maar soms ook wordt waargenomen bij kankercellen. Cellen die EMT ondergaan verliezen epitheliale kenmerken, en gaan steeds meer op mesenchymale cellen lijken. Zo verandert de vorm van de cel, verliezen de cellen hun onderlinge samenhang en wordt het voor de cellen mogelijk om los te komen van de primaire tumor. Hierdoor kunnen de cellen ingroeien in het onderliggend weefsel en zo via bloed- of lymfevaten metastaseren. Door EMT kunnen vaste tumoren dus losse, invasieve cellen vormen. Verschillende signaalroutes spelen een rol bij EMT, waaronder de Wnt en NOTCH-routes. Een van de grote aanstuurders van EMT is Transforming Growth Factor-β (TGF-β). In gezonde cellen is TGF-β een tumorsuppressor, maar in maligne cellen kan TGF-β juist cel-

motiliteit en angiogenese stimuleren. Ook L1CAM is een promotor van celmotiliteit die mogelijk een rol speelt in EMT. L1CAM wordt in steeds meer tumortypes gezien als een marker voor prognose. L1CAM is een membraanglycoproteine dat tijdens neurale ontwikkeling kan zorgen voor zowel celmotiliteit als celadhesie. In gezond weefsel wordt L1CAM met name tot expressie gebracht door zenuwweefsel, maar soms wordt het ook gevonden op het oppervlak van tumorcellen. Cellen gaan invasiever groeien wanneer tumoren in vitro worden blootgesteld aan L1CAM. Ook in vivo is dit proces herkenbaar, doordat L1CAM met name en soms zelfs alleen maar tot expressie wordt gebracht door de cellen aan de invasieve rand van tumoren. De rol van L1CAM is al onderzocht bij gynaecologische tumoren zoals endometrium- en ovariumcarcinoom, maar nog niet bij vulvacarcinoom.

Inhoud van dit proefschrift

de etiologie van HPV-positieve vulvacarcinomen wordt inmiddels goed begrepen, maar de HPV-onafhankelijke route moet nog worden ontrafeld. Dit proefschrift bevat resultaten van onderzoek waarmee wij proberen meer inzicht te verkrijgen in de ontstaanswijze van vulvacarcinoom.

In **hoofdstuk 2** wordt beschreven hoe een mutatieanalyse-panel werd ontwikkeld dat specifiek geschikt is voor gynaecologische maligniteiten. Dit panel richt zich op de meest relevante mutaties in cervix-, endometrium-, ovarium- en vulvacarcinoom. Om genetische mutaties in vulvacarcinomen te kunnen bestuderen werd een techniek geselecteerd (massaspectometrie) die geschikt is voor het kleine aantal patiënten met vulvacarcinoom en de daarbij behorende kleine hoeveelheid DNA-materiaal, dat vaak ook nog eens van matige kwaliteit is. Het is grotendeels nog onduidelijk welke genetische mutaties voorkomen in vulvacarcinomen. *TP53* is een bekende speler, maar mutaties in *CDKN2A* en *PTEN* zijn ook beschreven. De genen *HRAS*, *KRAS* en *NRAS* zijn eerder al bestudeerd in vulvacarcinomen, maar er werd tot nu toe nog geen mutatie in deze genen gevonden. Omdat er slechts kleine aantallen studies naar mutaties in vulvacarcinomen zijn verricht, hebben wij bij het samenstellen van het mutatiepanel gebruik gemaakt van kennis over mutaties bij vergelijkbare tumortypes, zoals gynaecologische tumoren, plaveiselcelcarcinomen van de huid, en hoofd-halstumoren. 171 somatische hotspot mutaties in 13 verschillende genen werden in het panel geïncludeerd. (*BRAF, CDKN2A, CTNNB1, FBXW7, FGFR2, FGFR3, FOXL2, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A* en *PTEN*). In totaal werden 564 tumoren (205 cervixcarcinomen, 227 endometriumcarcinomen, 89 ovariumcarcinomen en 25 vulvacarcinomen) gebruikt om het panel te testen en valideren. In 20% van de 25 vulvacarcinomen die in deze studie werden geanalyseerd werden mutaties gevonden in *CDKN2A*, *PIK3CA* en *HRAS*. Mutaties in *PIK3CA* en *HRAS* waren niet eerder beschreven bij vulvacarcinomen. Tegen verwachting in werden geen mutaties in *PTEN* gevonden. De re-

sultaten werden gevalideerd door enkele samples in duplo of in triplo te analyseren. Het panel bleek geschikt om grote aantallen samples tegelijk te bestuderen, was reproduceerbaar en bruikbaar voor paraffinemateriaal van lage kwaliteit en kwantiteit.

In **hoofdstuk 3** werd dit panel toegepast op een cohort van 108 patiënten met vulvacarcinomen. Deze patiënten werden tussen 2000 en 2009 behandeld voor een primair vulvacarcinoom in het Leids Universitair Medisch Centrum. Follow-up gegevens tot december 2012 werden verzameld uit patiëntendossiers. Hiermee is een groot cohort ontstaan, dat verder in dit proefschrift vaker gebruikt zal worden. Tumorweefsel van de patiënten in dit cohort werd onderzocht op aanwezigheid van HPV en middels massaspectrometrie onderzocht op de mutaties genoemd in het voorgaande hoofdstuk (*BRAF, CDKN2A, CTNNB1, FBXW7, FGFR2, FGFR3, FOXL2, HRAS, KRAS, NRAS, PIK3CA, PPP2R1A* en *PTEN*). Daaraan werd het gen *TP53* toegevoegd dat middels Sanger sequencing werd bestudeerd. In 7 van de bovengenoemde genen werden mutaties aangetroffen. Van de 107 patiënten waarvan genoeg tumorweefsel was om mutatieanalyses te verrichten bevatten er 66 (62%) tenminste één mutatie. (*TP53* = 58, *CDKN2A*(p16) = 14, *HRAS* = 10, *PIK-3CA* = 7, *PPP2R1A* = 3, *KRAS* = 1, *PTEN* = 1). Zeventien procent van de tumoren in dit cohort was HPV-positief. Mutaties kwamen het meest voor bij HPV-negatieve tumoren. De 5-jaarsoverleving was significant slechter voor patiënten mét een mutatie (47% versus 59%, *P* = 0.035). Het duidelijkst was dit verschil bij patiënten met een *HRAS*-mutatie.

In **hoofdstuk 4** werden de uitkomsten van hoofdstuk 3 vergeleken met de huidige kennis over genetische en epigenetische veranderingen in vulvacarcinomen en de voorlopers van vulvacarcinomen. Deze literatuurstudie liet zien dat veel onderzoekers hebben aangetoond dat genetische mutaties vaker voorkomen bij HPV-negatieve vulvacarcinomen dan bij HPV-positieve vulvacarcinomen. Het gen dat het meest gemuteerd was is *TP53*. 'Allelic imbalance' en 'loss of heterozygosity' werden vaker gevonden in hogere stadia van dysplasie en invasieve carcinomen en kwam voor bij zowel HPV-positieve als HPV-negatieve tumoren. Een klein aantal studies beschrijft epigenetische veranderingen in vulvaire laesies. Hypermethylatie werd het meest bestudeerd en dan met name hypermethylatie van het gen *CDKN2A*.Voor de meeste genen geldt dat hypermethylatie vaker voorkomt in carcinomen dan in voorloperlaesies.

De meerderheid van vulvacarcinomen zijn plaveiselcelcarcinomen, en binnen dit type kanker kunnen morfologische subclassificaties worden gemaakt die kunnen helpen te voorspellen en begrijpen wat de prognose voor patiënten is. In **hoofdstuk 5** beschrijven we het spoelcellige of spindle cell carcinoom, een subtype van plaveiselcelcarcinoom. Dit subtype wordt in verschillende soorten kanker beschreven en geeft in plaveiselcelcarcinomen van de long, slokdarm en het hoofd-halsgebied een slechtere prognose. Spindle cellen hebben hun epitheliale

kenmerken deels verloren en lijken makkelijker door te dringen in onderliggende weefsels. Het cohort van 108 patiënten werd onderzocht door middel van keratinekleuringen op weefselcoupes. In 20% van de gevallen bleek een component met spoelvormige cellen aanwezig. Deze patiënten hadden een slechtere prognose, met een 5-jaarsoverleving van 27.3 versus 58.2% (*P* = 0.00041) en een verhoogd risico op lymfekliermetastasen (relatief risico 2.26 met een 95% confidence interval van 1.47-3.47). Ook ziekte-specifieke overleving was significant slechter voor patiënten met spindle cell morfologie (45.2% versus 79.7% 5-jaarsoverleving, *P* = 0.00057). Wanneer gecorrigeerd werd voor mogelijke confounders bleek spindle cell morfologie een onafhankelijke prognostische factor voor overleving, met een hazard ratio van 4.1 (95% CI 1.61-10.60) Dit tumortype lijkt in sommige gevallen te ontstaan onder invloed van EMT, een proces dat onder invloed staat van onder andere L1CAM.

In **hoofdstuk 6** werd de prevalentie en prognostische waarde van L1CAM onderzocht in twee cohorten uit het Leids en het Groningse Universitair Medisch Centrum. L1CAM kan de celmotiliteit in tumoren stimuleren en blijkt bij steeds meer kankertypes een prognostische rol te spelen. Het Leidse cohort van 103 geschikte tumorweefsels en een tweede cohort uit Groningen van 245 primaire vulvacarcinomen werden gekleurd voor L1CAM, vimentine en E-cadherine. Het Leidse cohort was eerder al onderzocht op HPV-infectie en de aanwezigheid van *TP53* en *CTNNB1* mutaties. L1CAM werd gezien in het invasieve front van 17% van alle tumoren die werden onderzocht. L1CAM-positieve tumoren brachten vaker vimentine tot expressie, wat een maat is voor verlies van epitheliale kenmerken. L1CAM expressie was niet gecorreleerd met *TP53* of *CTNNB1* mutaties. De 5-jaarsoverleving was slechter voor patiënten met L1CAM-expressie (overall overleving 46.1% vs 63.6%, *P*=.014, ziekte specifieke overleving 63.8% vs 80.0%, *P*=.018). Multivariate analyse wijst L1CAM expressie aan een onafhankelijke prognostische marker is voor overleving (HR 2.9, 95% CI 1.10–7.68). Een in vitro assay met sferoïden van door ons gekweekte L1CAM-positieve vulvacarcinoomcellen laat zien dat de mate van invasieve groei afneemt wanneer L1CAM geneutraliseerd wordt.

In **hoofdstuk 7** werd de relatie tussen genetische mutaties en morfologische kenmerken onderzocht. Inmiddels is aangetoond dat patiënten met spindle cell carcinomen een hoger risico op lymfekliermetastasen hebben. Het is echter nog niet duidelijk of het de spoelvormige cellen zelf zijn die naar de lymfeklieren metastaseren. Als de spindle cel component van een tumor een uniek mutatieprofiel heeft dat verschilt van dat van de solide component in de primaire tumor, kan dit profiel gebruikt worden om te onderzoeken welke component van de tumor metastaseert of recidiveert. In dit hoofdstuk werden 66 tumoren uit het basiscohort geselecteerd die een lymfekliermetastase of een recidief hadden. Om spindle cellen te isoleren uit de andere tumorcellen en gezond weefsel werd gebruik gemaakt van Fluorescence Activated Cell Sorting. Alle samples werden onderzocht

op de aanwezigheid van een mutatie in de genen *BRAF*, *CDKN2A*(p16), *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A* en *PTEN*. Er was geen verschil in mutatiefrequentie tussen vulvacarcinomen met of zonder spindle cell morfologie (775 versus 58%). Ook in recidieven en metastasen bleken *TP53*, *CDKN2A* en *HRAS* het meest gemuteerd. De mutatieprofielen van de primaire tumor waren in 58% verschillend van de lymfekliermetastasen en in 71% van de recidieven. Het mutatieprofiel van spindle cell componenten leek vaker op dat van de solide groeiende cellen in de primaire tumor dan metastasen en recidieven, maar was in 14% van de gevallen toch verschillend van de solide component in de primaire tumor. Mutaties die primaire spindle cellen konden verbinden met recidieven of metastasen werden niet gevonden. Hiermee konden we niet aantonen dat er een selectieve rol is voor genetische mutaties in het ontstaan van spindle cell carcinomen of voor progressie naar metastasen en recidieven. Wel laat dit onderzoek de aanwezigheid van verschillende klonen binnen een tumor zien, die ieder een eigen spectrum van mutaties bij zich dragen. Het is van belang bewust te zijn van klonaliteit van tumoren, omdat bij gerichte therapie selectie kan plaatsvinden van klonen die een afwijkend spectrum hebben ten opzichte van de rest van de tumor. Vrijwel alle vulvacarcinomen met spindle cell morfologie bleken bij FACS analyse aneuploid.

De gegevens die bij de totstandkoming van dit proefschrift zijn verzameld hebben ons nieuwe inzichten gegeven in het gedrag van vulvacarcinomen. We hebben bevestigd dat HPV gerelateerde plaveiselcelcarcinomen van de vulva in de basis een ander type tumor is dan plaveiselcelcarcinomen van de vulva die zijn ontstaan vanuit genetische mutaties. Dat *TP53* een belangrijke speler is staat buiten kijf. In dit proefschrift wordt aangetoond dat er meerdere andere mutaties zijn die bijdragen aan het ontstaan en de progressie van vulvacarcinomen.

We hopen dat de resultaten in dit proefschrift een begin zullen zijn van een nieuw hoofdstuk in het onderzoek naar vulvacarcinomen, en dat dit nieuwe mogelijkheden zal bieden waarmee we vulvacarcinoom beter kunnen begrijpen, maar vooral ook beter kunnen behandelen.

Curriculum Vitae

Marjolijn Dorothea Trietsch werd geboren op 22 oktober 1983 in Den Haag. In 2002 behaalde Jolijn haar VWO diploma aan het Dalton Den Haag. Middels decentrale selectie werd zij in datzelfde jaar toegelaten tot de studie Geneeskunde in Leiden. Van 2005 tot 2006 onderbrak zij haar studie om een jaar als StudentAssessor van het LUMC te werken. Hieruit vloeiden lidmaatschappen van diverse besturen en commissies voort, waaronder van de Studentenraad, het Landelijk Medisch Studenten Overleg en het Landelijk Overleg CoAssistenten. In 2007 verbleef zij enkele maanden in Suriname om daar de co-schappen Dermatologie en Keel- Neus- en Oorheelkunde te volgen in het Academisch Ziekenhuis Paramaribo. Haar afstudeerstage naar warmtetherapie van condylomata accuminata bij HPV-positieve mannen vond plaats op de afdeling Gynaecologie / Pathologie. Binnen een samenwerking tussen de afdelingen Gynaecologie, Pathologie en het Female Cancer Program (thans Female Cancer Foundation) begeleidde zij een uitwisselingsproject met Indonesische studenten. In 2009 begon Jolijn onder leiding van professor G.J. Fleuren en professor A.A.W. Peters aan het promotietraject op de afdeling Pathologie waaruit dit proefschrift is ontstaan. Hiernaast volgde zij de opleiding tot epidemioloog A. In 2014 en 2015 werkte zij als arts-assistent niet in opleiding voor de afdelingen Heelkunde, Urologie en Gynaecologie / Verloskunde in het Medisch Centrum Haaglanden in Voorburg en Den Haag. In juli 2015 begon zij aan de opleiding tot Gynaecoloog in het Reinier de Graaf Gasthuis in Delft (Opleider C.J. Kapiteijn) en het Leids Universitair Medisch Centrum (Opleider J.M.M. van Lith).

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Dankwoord

Een proefschrift schrijven doe je nooit alleen.
Dank aan iedereen die hulp, vertrouwen, inzicht, een luisterend oor, gezelligheid, advies, relativering, frituur, kennis, ervaring, afleiding, prikkeling, feedback, energie, kameraadschap en vooral geduld heeft gegeven.
Zonder jullie was dit niet gelukt.