

SQUAMOUS EPITHELIAL LESIONS OF THE VULVA

REFINING HISTOLOGICAL
DIAGNOSTIC CRITERIA
AND EXPLORING
BIOMARKERS

SHATAVISHA DASGUPTA



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Refining histological diagnostic criteria
and exploring biomarkers

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Squamous Epithelial Lesions of the Vulva

**Refining histological diagnostic criteria
and exploring biomarkers**

Plaveiselcel laesies van de vulva
verfijning van histologische diagnostiek criteria en mogelijke biomarkers

Dissertation

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Erasmus University Rotterdam
by command of the rector magnificus,
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By

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Born in West Bengal, India

Erasmus University Rotterdam

The Erasmus University logo, featuring a stylized, handwritten-style script of the word "Erasmus" in a dark, possibly black, ink.

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Dedicated to the loving memory of my grandparents,
Mr. Prafulla Sen and Mrs. Runu Sen.

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CHAPTER 1

GENERAL INTRODUCTION AND SCOPE OF DISSERTATION

1. General introduction

The vulva, or the female external genitalia, has a complex anatomy and histology that change throughout a woman's life cycle [1]. The vulva can be affected by a spectrum of benign, pre-malignant, and malignant lesions, around 90% of which arise from the squamous epithelial lining [2]. When patients present with symptoms suggestive of a vulvar lesion, e.g. a growth, or persistent itching or irritation, the clinician may remove a sample of tissue from a representative area for pathological assessment [3]. Definitive diagnosis of the lesion established on the pathological assessment informs the treatment and follow-up strategies [4]. Benign lesions can be treated with topical medications, cryotherapy, or a local excision, whereas, for pre-malignant and malignant lesions, excision with microscopically disease-free margins is the treatment of choice [5-7]. Quite evidently, accurate and reliable pathological diagnosis is crucial to allow appropriate treatment.

Pathological diagnosis of vulvar lesions, however, can be challenging, as the histological appearances of some of these lesions tend to overlap [8]. As a result, there can be discordances among pathologists for the diagnosis, or in the interpretation of the histological features [9]. The aim of this dissertation is to facilitate the diagnosis of vulvar squamous lesions by – (i) refining the histological diagnostic criteria, and (ii) exploring biomarkers (immunohistochemical or molecular) that may be used as diagnostic adjuncts. In the following sections, the anatomy and the histology of the normal vulva is described, a brief overview of the lesions addressed in the dissertation is presented, and the scope of the dissertation is discussed.

1.1 *Vulvar Anatomy*

The vulva consists of anatomical structures of both the reproductive and the urinary tract [10]. Externally, the vulva is covered by skin-folds, called the labia majora and the labia minora. The labia majora are the larger, hair-bearing skin-folds that fuse anteriorly into the mons pubis, and posteriorly into the perineum. The labia majora cover the labia minora, which are smaller and non-hair-bearing. The space between the labia majora and minora is called the inter-labial sulcus [Figure 1].

The vulva also comprises the clitoris and the vestibule. The clitoris functions as a sensory organ, and is homologous to the glans penis in males. The vestibule is the area between the labia minora and contains the urethral opening, introitus or the vaginal opening, Bartholin's glands or the greater vestibular glands, and Skene's glands or the lesser vestibular glands.

1.2 Vulvar Histology

The outer part of the vulva, i.e. the labia majora and the minora, are lined by keratinized, stratified squamous epithelium [1, 10]. The lateral part of the labia majora and the mons pubis contain sebaceous glands, eccrine glands, and apocrine sweat glands [Figure 2].

The keratinized epithelium of the labia transitions into non-keratinized squamous epithelium of the vestibule at the imaginary Hart's line. Within the vestibule, the urethral and the vaginal openings are lined by glycogen-rich stratified squamous epithelium. Bartholin's and Skene's glands are lined by mucous-secreting columnar epithelial cells.

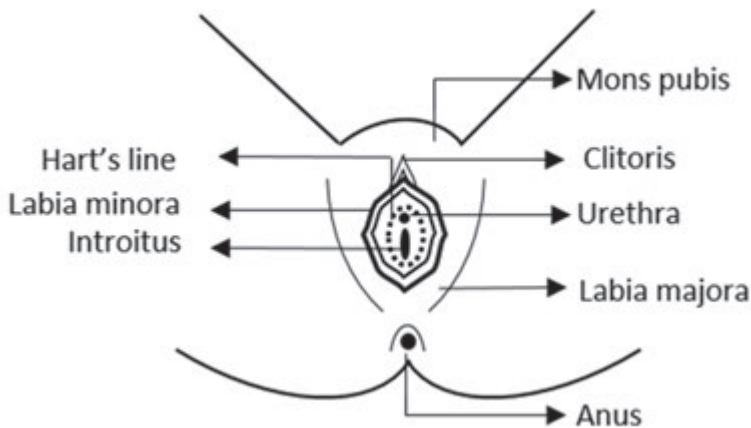


Figure 1: Graphical representation of vulvar anatomy

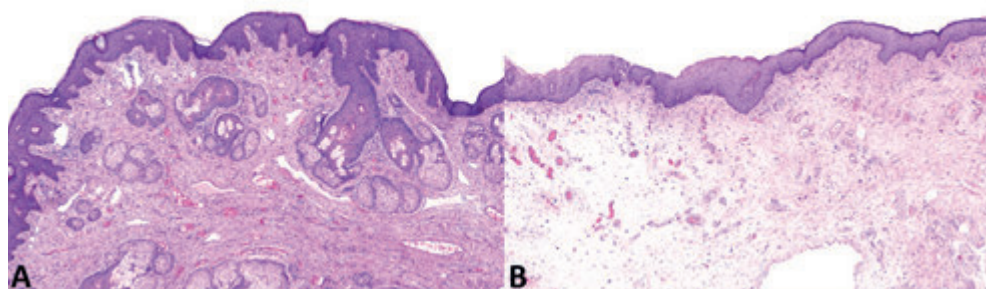


Figure 2: Histological appearance of (A) normal hair-bearing vulvar skin, and (B) normal non-hair-bearing vulvar skin (hematoxylin-eosin staining, original magnification 5X)

1.3 Vulvar Pathology

1.3.1 Benign lesions

Common benign lesions of the vulva include vulvar seborrheic keratosis (VSK) and condyloma acuminata. Both lesions can develop on hair-bearing skin of the vulva, and are known to have some similarities in their histological appearances. For condyloma, an etiological role of low-risk (LR) human papillomavirus (HPV) is well-established [11]. In contrast, for VSK, the association with HPV is considered contentious [12].

Although LR-HPV has been detected in VSKs in multiple studies, there has been debate whether this could be attributed to incidental detection of resident HPV [13], or whether these studies erroneously included condylomas due to the use of non-stringent histological criteria [14]. To date, the association of HPV and genital SKs remains controversial.

Determining the exact relationship of HPV with VSKs is not merely of academic interest, as the HPV-status of a lesion may have bearing on the management decision [15, 16]. In general, VSKs do not warrant treatment, and are usually removed when symptomatic [17]. Where there is an association with HPV, treatment of VSKs will need to be directed to addressing the viral cause, in order to prevent lesional recurrence or unnecessary surgical procedures [15].

Clarity on the association of HPV and VSK may be obtained by performing HPV-testing and immunohistochemistry (IHC) with cellular and viral biomarkers on VSKs selected following stringent histological criteria.

1.3.2 Pre-malignant and malignant lesions

Vulvar squamous cell carcinoma (VSCC) accounts for < 5% of all gynecological malignancies, but for almost 90% of all vulvar malignancies [4]. Since the 1990s, an increase (18%) in the incidence of VSCC has been documented [3]. Although traditionally considered to be a disease of women of > 70 years of age, age-standardized incidence of VSCC in women of 50 – 59 years of age has increased by 92% in recent years [4]. In the Netherlands, around 385 new cases of VSCC are diagnosed every year, and over the past decade, increased incidence has been observed in both younger and older women [18].

Increasing evidence has established that VSCC can arise via two distinct etio-pathological pathways – HPV-related and HPV-independent [19]. Only 25% of VSCCs arise via the HPV-related pathway, whereas, the majority (75%) arise via the HPV-independent pathway [19-21].

HPV-related VSCC, also called HPV-associated VSCC according to the 5th edition of the WHO Classification of female genital tumors, usually affects women in the 3rd – 5th decades of life, and are caused by high-risk (HR)-HPV genotypes, most commonly, HPV16 [2]. The viral onco-proteins E6 and E7 inactivate and degrade tumor suppressor proteins, p53 and RB1, thereby up-regulating the cell-cycle and causing uncontrolled cell-proliferation [22]. In contrast, HPV-independent VSCC affect women in the 6th – 8th decades of life, and has been associated with long-standing dermatoses, such as lichen sclerosus (LS), or, less frequently, lichen planus (LP) [23, 24]. Somatic mutations of *TP53* have been detected in 80% of HPV-independent VSCC, however, the exact mechanism of the pathogenesis is poorly understood. The two subtypes of VSCC also differ in terms of their clinical behavior – HPV-independent VSCC has a worse prognosis than HPV-related VSCC (5-year survival 68% vs. 93%) [25]. Current management strategies of VSCC are not dictated by the etio-pathological subtype [4, 26]. The mainstay of treatment for both subtypes is surgical excision with tumor-free margins, with or without inguinal lymph node dissection [4, 26].

Both subtypes of VSCC have been postulated to arise via a premalignant stage, known as, vulvar intraepithelial neoplasia (VIN) [19, 20]. For HPV-related VSCC, the precursor lesion is named squamous intraepithelial lesion, HPV-associated in the latest edition of WHO classification. Other accepted terms are high-grade squamous intraepithelial lesion (HSIL), or, usual VIN (uVIN), which are used in this dissertation [19, 20]. For HPV-independent VSCC, the most well-

characterized precursor lesion is named vulvar intraepithelial neoplasm, HPV-independent, in the latest edition of the WHO classification. Other accepted term is differentiated vulvar intraepithelial neoplasia (dVIN), which is used in this dissertation [19, 20]. However, recent studies suggest that additional putative precursors of HPV-independent VSCC may exist, namely, vulvar acanthosis and altered differentiation (VAAD), and differentiated exophytic vulvar intraepithelial lesion (de-VIL) [27-30]. As yet, there is limited information available on the clinical and histological characteristics of VAAD and de-VIL.

HSIL and dVIN have distinct clinical and pathological characteristics. On histology, HSIL usually shows a basaloid appearance, with conspicuous, full-thickness architectural and cytological atypia, and occasional presence of koilocytes [19, 20]. Unlike HSIL, histological appearance of dVIN can be subtle, often resembling the reactive changes of lichenoid dermatoses. The most characteristic feature of dVIN is the paradoxical maturation due to abnormal keratinization, and nuclear atypia is commonly confined to the basal / parabasal layers [31]. As a result, dVIN can be under-diagnosed by pathologists [32]. A retrospective Dutch study detected dVIN in 42% of cases previously diagnosed on histology as LS that progressed to VSCC [32]. Inability to accurately discriminate dVIN from reactive dermatoses therefore, may result in missed opportunities for preventing progression to VSCC in these 42% of cases.

Recent literature also suggests that dVIN and HSIL can occasionally mimic each other on histology. dVIN showing a basaloid appearance and full-thickness atypia, i.e. HSIL-like dVIN, and HSIL showing keratinization, i.e. dVIN-like HSIL, have been reported [31]. However, distinguishing between dVIN and HSIL on pathological assessment is crucial because dVIN has a higher chance (6-fold) and faster rate of progression to VSCC (23 months vs. 41 months) [33]. Thus, when faced with the diagnosis of a pre-malignant lesion, the pathologist needs to determine – (i) whether the lesion is dysplastic (i.e. VIN or no-VIN), and (ii) if VIN, then whether it is dVIN or uVIN (HSIL). Well-defined and reproducible histological diagnostic criteria is therefore essential.

In pathology practice, IHC is the most widely used tool to complement difficult histological diagnoses. Conventionally, for dVIN and HSIL, IHC with p16 and p53 is used [31]. With p16, HSIL typically shows block-type expression, i.e. confluent, strong nuclear and / or cytoplasmic staining involving $\geq 1/3$ rd of the epithelium, which is a probable consequence of cell-cycle deregulation caused by E6 and E7

onco-proteins [34]. Therefore, block-type p16-expression is considered to be a reliable surrogate marker of HR-HPV-infection, and supportive of a diagnosis of HSIL [34]. With p53, HSIL shows wild-type expression, which can either be scattered expression in the basal layers, or scattered expression in the mid-epithelial layers, with sparing of the basal layers [35]. In contrast, dVIN either shows no expression or non-block-type expression with p16, and mutant patterns (overexpression / null) of expression with p53, which has been reported to reflect underlying *TP53* mutations that characterize dVIN [36].

However, p53 IHC may not inform the diagnosis of dVIN in every case. A proportion of dVIN has been reported to show wild-type expression, while, reactive dermatoses have been occasionally reported to show overexpression [31]. Moreover, p53 IHC does not contribute to the diagnosis of the novel putative precursors, VAAD and de-VIL, as these have also been reported to show wild-type expression [36]. Identification of novel biomarkers can be helpful for the diagnosis of the precursors of HPV-independent VSCC that show wild-type p53-expression. Since no single immunohistochemical marker is likely to have perfect sensitivity and specificity, using markers in a panel may help in reaching a gold-standard diagnosis.

Identification of novel biomarkers, immunohistochemical or molecular, is also relevant for improving the diagnosis or treatment of VSCC [37]. In recent years, for several malignancies, advancement and personalization of treatment have been achieved by identifying targetable genetic and epigenetic changes. Similar progress in VSCC treatment has not been possible due to the lack of molecular studies, thereby earning VSCC the monikers – ‘forgotten women’s cancer’ and ‘Cinderella of Gynecological Oncology’ [38, 39]. Currently, for VSCCs, surgical excision with curative intent is the mainstay of treatment. However, due to the anatomical complexity of the vulvar region, patients with VSCC frequently suffer post-surgical morbidities, such as urinary and fecal incontinence, that adversely impact the quality of life. A better understanding of the molecular underpinnings of VSCC may help identify biomarkers of potential diagnostic / predictive / prognostic / therapeutic significance.

2. Scope of the dissertation

This dissertation aims to refine histological diagnostic criteria and explore biomarkers for squamous epithelial lesions of the vulva, with a particular focus on pre-malignant lesions. The scope of this dissertation described below.

In **chapter 2**, we investigate the contentious relationship of HPV and VSK. For this, we evaluated the current evidence on this association by performing an in-depth review of the literature. In addition, to add to the existing evidence, we investigated the presence of HPV in a series of VSKs, using a novel combination of direct and surrogate approaches. This involved (i) both whole-tissue section based and laser-capture microdissection based polymerase chain reaction (PCR) for HPV-DNA detection and genotyping, and (ii) IHC with p16 and MIB-1 (cellular biomarkers), and E4 (viral biomarker), to augment HPV-testing.

Chapters 3 – 9 focus on pre-malignant and malignant lesions of the vulva.

In **chapter 3**, we provide an overview of the current knowledge on the precursor lesions of VSCC and the biomarkers (immunohistochemical and molecular) that have been studied for these lesions. With a view to identifying additional potential biomarkers for VSCC and its precursor lesions, we also analyzed publicly available gene expression datasets.

In **chapter 4**, we established the most helpful diagnostic features of dVIN, by reviewing the histology of a large retrospective series of dVIN, and its closest histological differential, LS. In addition, we explored the usefulness of two immunohistochemical markers, cytokeratin 13 (CK13) and cytokeratin 17 (CK17) for the diagnosis of dVIN, in particular for discriminating dVIN and LS.

In **chapter 5**, we evaluated the inter-observer agreement for the diagnosis, and in the interpretation of histological features of dVIN, among a bi-national, multi-institutional group of pathologists. Our aim was to investigate whether the histological features that were identified as helpful for diagnosing dVIN in chapter 4, could be reproducibly interpreted by pathologists in the real-world. We also assessed the perception of the pathologists regarding the diagnostic usefulness of the individual histological features.

In **chapter 6**, we evaluated immunohistochemical markers CK17 and SOX2, as adjuncts to p53 for the diagnosis of dVIN, by comparing the expression of these markers in dVIN, de-VIL, HSIL, and non-dysplastic vulvar tissue. In addition, to

facilitate the identification of other potential diagnostic markers, we performed next generation targeted sequencing on a subset of dVIN and de-VIL.

In **chapter 7**, we identified differentially expressed genes (DEGs) in VSCC, by analyzing two independent gene-expression datasets, one from gene expression omnibus and one from a previous study at our center. From both datasets, we identified a set of DEGs that were similarly regulated (up or down) in VSCC (HPV-related and HPV-independent) with statistical significance. We studied immunohistochemical expression of two of these DEGs in VSCC, dVIN, HSIL, and normal vulvar tissue.

In **chapter 8**, we investigated the differences in DNA-methylation patterns of VSCC and normal vulvar tissue, to identify markers of potential diagnostic / predictive / prognostic / therapeutic significance.

In **chapter 9**, we aimed to identify clinical and pathological factors that may predict the final diagnosis of VSCC, for women who underwent surgical treatment based on strong clinical suspicion, despite the lack of a conclusive pre-operative diagnosis of VSCC.

Chapter 10 is the general discussion addressing the findings of this dissertation in the context of current knowledge.

Chapter 11 summarizes the findings of this dissertation, and outlines future perspectives.

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2



CHAPTER 2

RELATIONSHIP OF HUMAN PAPILLOMAVIRUS WITH SEBORRHEIC KERATOSIS OF THE FEMALE GENITAL TRACT – A CASE-SERIES AND LITERATURE REVIEW

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Under review in Histology and Histopathology

Abstract

Seborrheic keratoses (SKs) are benign lesions of uncertain etiology, which can develop in both genital and extra-genital locations. For genital SKs, there has been conjecture about the pathogenic role of human papillomavirus (HPV), in view of the frequent association of this virus with genital lesions.

In light of the potential consequences on patient management, we investigated the relationship between HPV and SKs of the female genital tract (FGT). For this, we evaluated the current evidence on this relationship by performing an in-depth review of the literature. Furthermore, to add to the evidence on this association, we investigated the presence of HPV in a series of vulvar SKs (n = 15), using a novel multimodal approach. This involved whole tissue section-polymerase chain reaction (WTS-PCR) using SPF10-DEIA-LipA25 for HPV detection and genotyping. In addition, immunohistochemistry (IHC) was performed with cellular biomarkers p16 and MIB-1, and viral biomarker E4, to augment HPV-testing. Finally, laser-capture microdissection-PCR (LCM-PCR) was performed to locate HPV to specific lesional cells, and to rule out incidental detection of resident HPV with WTS-PCR.

Our findings from the literature review, as well as, the case-series are presented.

1. Introduction

Seborrheic keratoses (SKs) are benign, wart-like epidermal lesions, prevalent in middle-aged or elderly individuals of both sexes [1]. These lesions can develop on any part of the body, but are relatively uncommon in the genital area [2]. Although sun-exposure and aging have been postulated to be risk-factors, the exact mechanism of the pathogenesis of SKs remains unknown.

For genital SKs, the role of human papillomavirus (HPV) in the development has been previously investigated [3]. This is in view of the frequent association of HPV-infection with primary epithelial lesions of the genital tract, and the histological similarity of SKs with the low-risk(LR)-HPV-related lesion, condyloma acuminata [3-5]. HPV was detected in genital SKs in multiple studies, however, there has been debate on the significance of this finding. Some researchers argued whether these results could be attributed to incidental detection of resident HPV, which is part of the microbiological flora of normal skin [6]. Others commented that these studies could have erroneously included condylomas, instead of SKs, due to the use of non-stringent histological criteria [4]. To date, the association of HPV and genital SKs remains controversial.

Determining the exact relationship of HPV with genital SKs is not merely of academic interest, as the HPV-status of a lesion may have bearing on management decisions [7,8]. In general, SKs do not warrant treatment, and are usually removed when symptomatic, or for cosmetic reasons [9]. Where there is an association with HPV, treatment of SKs will need to be directed to addressing the viral cause, in order to prevent lesional recurrence or unnecessary surgical procedures [8].

Through this study, we aimed to establish the association between HPV and SKs of the female genital tract (FGT). For this, we evaluated the current evidence on this association by performing an in-depth review of the literature. In addition, to add to the existing evidence, we investigated the presence of HPV in a series of vulvar SKs (VSKs), using a novel combination of direct and surrogate approaches. This involved (i) whole tissue section-polymerase chain reaction (WTS-PCR) for HPV detection and genotyping; (ii) immunohistochemistry (IHC) with cellular biomarkers p16 and MIB-1, and the viral biomarker E4, to augment HPV-testing; and (iii) laser-capture microdissection-PCR (LCM-PCR) to locate HPV to specific lesional cells. Where HPV was detected, we investigated whether the patient had a recent history of other HPV-related vulvar lesions, and performed WTS-PCR and IHC on these for comparison.

2. Materials and Methods

2.1 Literature review

Electronic search strategies combining Medical Subject Headings (MeSH) and free-text words were prepared with the help of medical librarians at Erasmus MC. Biomedical bibliographic databases, namely, Embase.com, MEDLINE (Ovid), Cochrane Central Register for Controlled Trials (Wiley), Web of Science Core Collection (Web of Knowledge), and Google Scholar were searched. The full search strategy is provided in supplement 1. The last search was conducted in December, 2020.

A total of 374 unique references were identified from the databases. These were screened by reading the title and / or abstract by one author (SDG). Original and review articles that met the following criteria were included – (i) abstract available; (ii) written in English language; and (iii) reporting on SKs of the FGT. Case reports, conference abstracts, animal studies, and in-vitro studies were excluded.

Thirty-nine references were included after the first round of screening. These references were screened by two authors (SDG and BM) to specifically identify studies that fulfilled the inclusion criteria. Five such references were found and for all of these, full text was available. Five additional studies were included to prepare the narrative synthesis. The process of reference selection is depicted in Figure 1.

2.2 Case-series

Lesions that had been histologically diagnosed as VSKs (2009 – 2018) were retrospectively identified from Erasmus MC, and Ikazia Hospital, Rotterdam. Hematoxylin-eosin (HE) stained slides were retrieved from the archives and reviewed by two pathologists (SDG and PCEG). Relevant clinical information was gathered from the patient records. All patient data were anonymized.

For WTS-PCR, sections were prepared from the formalin-fixed paraffin embedded (FFPE) tissues using a sandwich method to yield one 4µm-thick section for HE-staining (HE-before); two 3µm × 8µm sections for WTS-PCR; three 4µm-thick sections for IHC; and one 4µm-thick section for HE-staining (HE-after). The HE-before and HE-after slides were independently reviewed by an experienced pathologist (MvdS) to confirm the histological diagnoses.



PRISMA 2009 Flow Diagram

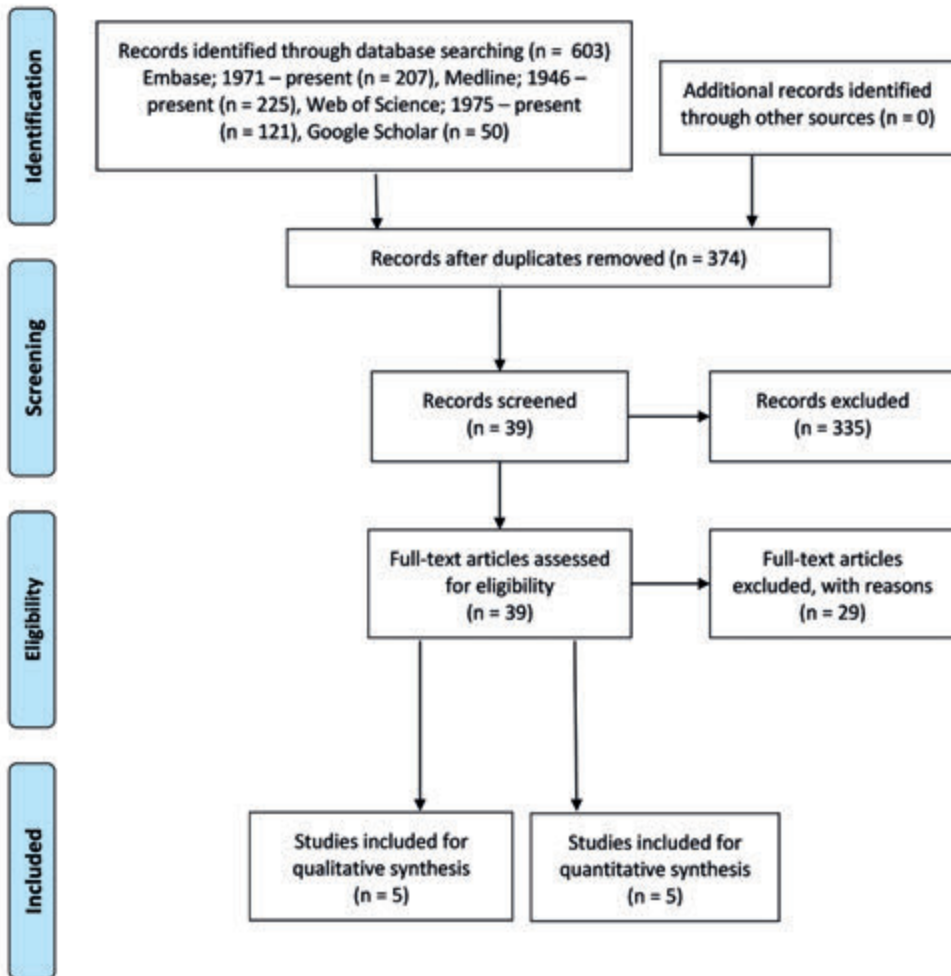


Figure 1: PRISMA flow diagram depicting the process of literature inclusion

2.2.1 WTS-PCR

WTS-PCR was performed using the SPF10-DNA Enzyme Immunoassay (DEIA)-Line Probe Assay (LiPA) 25 system (Labo Bio-Medical Products, The Netherlands), containing probes for 25 different HPV-genotypes (HPV6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74).

2.2.2 IHC

IHC was conducted with p16 using the ready-to-use primary mouse monoclonal antibody (mAb) clone E6H4 (Ventana), with MIB-1 using the clone Ki-67 (Ventana), and with E4 using the PanHPVE4 mAb XR-E4-1 (SILgrade-E4 kit, Labo Bio-Medical Products). IHC was scored as follows:

p16: complete lack of staining = no expression; nuclear and / or cytoplasmic staining in non-contiguous cell clusters extending less than 1/3rd of the epithelial thickness = non-block-type / patchy expression; nuclear and/or cytoplasmic expression extending from the basal layer through at least 1/3rd of the epithelial thickness [10] = block-type expression.

MIB-1: nuclear staining in a single or double row of cells only in the basal / parabasal layers = negative; at least two moderately to strongly stained nuclei within the same high-power field (400X magnification) in the upper 2/3rd of the epithelium = positive [11,12].

E4: no staining = 0; focal staining restricted to groups of cells in the upper epithelial layers = 1; extensive staining in the upper half of epithelium or more = 2. Score ≥ 1 was interpreted as positive [13].

2.2.3 LCM-PCR

Regions for LCM-PCR were selected on the digital scans (Aperio Inc., CA) prepared from the glass-slides. Areas of these regions ranged from 30,262 – 74,322 μm^2 (mean = 57,936 μm^2). These were excised using Zeiss PALM microbeam UV-laser-microdissection system, and thereafter analyzed using SPF10-DEIA-LiPA25. Methodology of WTS-PCR, IHC, and LCM-PCR is detailed in supplement 2.

3. Results

3.1 Literature review

Information extracted from the five studies that investigated the association of lesions having the histology of SKs of the FGT and HPV are presented in Table 1, and summarized in the following sections.

3.1.1 Prevalence of HPV in SK of female genital tract (FGT)

Of the 5 previous studies, 2 were on VSKs [14,15], 2 were on VSKs, pubic and perianal SKs [2,3], and 1 was on cervico-vaginal SK-like lesions [5]. In 3 of these studies, non-SK genital lesions, comprising benign (85%), pre-malignant (11%), and malignant lesions (4%) constituted the control group.

Mean age of patients with SKs (n = 88) was 47.8 years (range: 5 – 80 years). For all SKs, the median prevalence of HPV was 57.5% (range: 14.2 – 75%; mean: 51%), while for VSKs, this was 46% (range: 14.2 – 100%; mean: 51.5%). Low-risk (LR)-HPV6 was detected most frequently (72%), followed by rarer untypable HPV-genotypes (12%), LR-HPV11 (3%), and high-risk (HR)-HPV16 (3%).

Mean age of the patients with non-SK genital lesions (n = 67) was 47.9 years (range: 9 – 70 years). In 1 study, condyloma acuminata was studied as the non-SK lesion, and HPV was detected in all of these (genotyping not performed). For the rest of the non-SK genital lesions, median prevalence of HPV was 0% (range: 0 – 9.1%; mean: 3.3%), and the detected HPVs comprised LR-HPV6 and HR-HPV16.

3.1.2 Histology

A summary of the histological description of the SKs extracted from the included references is presented below.

The lesions are circumscribed or polypoid, having a stuck-on configuration, composed of broad, coalescing sheets, islands, and interconnecting trabeculae of cells. Lesional cell nests are surrounded by scant fibrovascular stroma with prominent hyaline basement membrane-like material and ectatic, thin-walled vessels. The lesional cells have a bland, basaloid appearance. Nuclei are uniform, ovoid to spindle-shaped, with occasional nuclear grooves. Mitotic count is generally low. Areas of peripheral palisading, hyperkeratosis, basket-weave or laminated orthokeratosis, papillomatosis, acanthosis, variable melanin

pigmentation, horn pseudo-cysts, and squamous eddies are characteristic features. Clusters of cells with clear cytoplasm can be occasionally present. Viral cytopathic changes e.g. koilocytes, parakeratosis, compact orthokeratosis, or hypergranulosis are typically absent.

Reutter et al. reported parakeratosis to be a specific predictor of the presence of HPV in VSKs [14]. In contrast, Bai et al. and Tardio et al. observed that HPV-status of SKs could not be reliably predicted from any particular histological feature [2,15].

3.1.3 IHC

p16: Two studies [5,15] investigated p16-expression in SKs. In the series of Bai et al., 11% (2/18) of the HPV-positive VSKs showed non-block-type / patchy p16-expression, while, the non-SK genital lesions did not show p16-expression. In the series of Talia et al., none of the HPV-positive SK-like lesions (n = 3) showed p16-expression, whereas, 75% (3/4) of the HPV-negative SK-like lesions showed a mosaic pattern of p16-expression.

MIB-1: One study [15] investigated MIB-1-expression in VSKs. MIB-1 positivity, i.e. nuclear staining in upper 2/3rd of epithelial thickness, was observed in 72% (18/25) of HPV-positive VSKs, and showed good concordance with the HPV-status (kappa = 0.42). In the same study, 22.7% (5/22) of non-SK genital lesions also showed MIB-1-positivity, however, data on the concordance of MIB-1-expression and the HPV-status for these lesions were not presented.

Cyclin E: One study [15] investigated cyclin E-expression in VSKs. Cyclin E-positivity was observed in 76% of VSKs, and in 68.2% of non-SK genital lesions, and did not show any correlation with the HPV-status of the lesions.

Bai et al. reported that among p16, MIB-1, and cyclin E, the only biomarker that could augment HPV-testing for VSKs was MIB-1 [15].

Table 1: Studies that investigated the relationship between HPV and SKs of the female genital tract

References	Method of HPV-detection	Genital SKs			Genital non-SK lesions		
		Location (number)	Mean age (Range)	HPV prevalence; genotypes (frequency)	Lesion (number)	Mean age (Range)	HPV prevalence; genotypes (frequency)
Leonardi et al., 1991	HPV DNA PCR, Dot-blot and/or Southern blot	vulva (1); pubis (1); groin (1); buttock (1)	27.5 (5 – 44)	75%; HPV6 (2), HPV11 (1), HPV16 (1)	SF (6); BD (5); SD (4); LP (3); BCC (2); BH (1); CD (1); SCC (1); EA (1); Len. (1); DN (1); EN (1)	No data	0%; NA
Bai et al., 2003	HPV DNA PCR; RFLP	vulva (25)	46 (16 – 80)	72%; HPV6 (15), novel HPV (3)	NCA (10); FEP (12)	44.7 (25 – 70)	9.1%; HPV6 (1), HPV16 (1)
Tardio et al., 2012	HPV DNA PCR; hybridization	vulva (5); pubis (9); perianal (1)	42.7 (5 – 80)	33.3%; HPV6 (5)	AK (5); FEP (3); EC (2); Hem. (1); MN (1)	38.5 (9 – 54)	0%; NA
Reutter et al., 2014	HPV DNA PCR; RFLP	vulva (21)	67.5 (no data)	14.3%; HPV6 (2), novel HPV (1)	Cond. A (6)	60.7 (no data)	100%; genotyping not performed
Talia et al., 2017	HPV DNA PCR; hybridization	cervix (4); upper vagina (3); un-determined (1)	55.4 (41 – 70)	42.9%; HPV42 (3)		not studied	
Present series, 2021	HPV DNA PCR; reverse hybridization	vulva (15)	50.4 (25 – 70)	73.3%; HPV44 (5), HPV6 (4), HPV42 (2), HPV53 (1)		not studied	

SK: seborrheic keratosis; PCR: polymerase chain reaction; AK: angiokeratoma; EC: epidermal cyst; FEP: fibroepithelial polyp; hem.: hemangioma; MN: melanocytic nevus; SF: soft fibroma; BD: Bowen's disease; SD: superficial dermatitis; LP: lichen planus; BCC: basal cell carcinoma; BH: benign hyperplasia; CD: chronic dermatitis; SCC: squamous cell carcinoma; EA: epidermolytic acanthoma; Len.: lentigo; DN: dysplastic nevus; EN: epidermal nevus; NA: not applicable; RFLP: restriction fragment length polymorphism; NCA: non-condylomatous acanthoses; FEP: fibroepithelial polyp; AK: angiokeratoma; Hem.: hemangioma; MN: melanocytic nevus; cond. A: condyloma acuminatum

3.2 Case-series

Fifteen lesions were identified from 13 patients of ages between 25 – 70 years (median = 52 years) [Table 2]. None of the patients were immunocompromised.

3.2.1 Histology

The lesions were acanthotic, composed of monomorphic keratinocytes having low nuclear-to-cytoplasmic ratio, arranged in coalescing sheets or in interconnected trabeculae, with areas of peripheral palisading [Figure 2]. Laminated and/or basket-weave orthokeratosis, horn pseudo-cysts, dilated capillaries within thickened papillary dermis, melanin pigment in the basaloid cells, and squamous eddies were frequently present. Mitotic figures were rare. Histological hallmarks of HPV-infection were absent.

3.2.2 WTS-PCR

HPV-DNA was detected in 11 (73%) VSKs (patients 5 – 13). The genotypes were LR-HPV6 (n = 4), LR-HPV44 (n = 4), LR-HPV42 (n = 2), probably-carcinogenic HPV53 (n = 1), and untypable (n = 1) [Table 3].

Patients 5, 6, and 10 had a history of vulvar high grade squamous intraepithelial lesion (HSIL), and patient 11 had a co-existing HSIL. HR-HPV16 was detected in HSILs from patients 5 and 10, and both LR-HPV6 and HR-HPV16 were detected in the HSIL from patient 11. Tissue for HPV-testing was not available for the HSIL from patient 6. Patient 5 developed a condyloma acuminatum on follow-up, in which LR-HPV6 was detected.

3.2.3 IHC

p16: Nine (82%) of the 11 HPV-positive VSKs showed extensive, non-block-type / patchy expression [Table 3; Figure 3]. All three HSILs showed block-type expression, and the condyloma showed extensive, non-block-type expression, similar to the VSKs.

MIB-1: All of the 11 HPV-positive VSKs were positive for MIB-1 [Table 3; Figure 3]. In 2 of these lesions, nuclear staining in the upper 2/3rd of the epithelium was present in fewer foci. In 1 lesion, nuclear staining was mostly present in the upper 2/3rd of the epithelium, with minimal staining in the basal layer. Of the 4 HPV-negative VSKs, 2 were MIB-1 positive. All three HSILs and the condyloma were MIB-1-positive.

Table 2: Clinical details

Pt.	Age* (y)	Specimen	H/O vulvar lesions	Macroscopic description	Co-existing vulvar lesions	Site	Follow-up (months)
1	67	Biopsy	SCC; dVIN	Pigmented, raised	None	Not specified	LS (26); thereafter NED (61)
2	53	Biopsy	None	Pigmented, raised	SCC	Lab. majus (R)	dVIN (5); anal hemorrhoids (16); radio-necrotic anal ulcers (34); thereafter NED (15)
3	66	Biopsy	None	Verrucous	SCC	Lab. majus (R)	Granulation tissue - vulva (5); thereafter NED (57)
4	52	Biopsy	SCC; HSIL	Skin tag-like	None	Lab. majus (L)	NED (12)
5	46	Biopsy	HSIL	Papillomatous, pigmented	None	Lab. minus (R)	Vulvar condyloma acuminatum (13); chronic inflammatory changes in vulva (28, 40); thereafter NED (17)
6	66	Excision	HSIL	Verrucous, pigmented	None	Lab. majus (R)	No FU
7	40	Biopsy	None	Papillomatous, pigmented	None	Lab. minus (R)	No FU
8	25	Excision	None	Papillomatous	None	Lab. majus (B/L)	CIN2 (1); Pap smear: NILM, HR-HPV+ve (9); Pap smear: NILM, HR-HPV-ve (19); thereafter NED (19)
9	52	Excision	None	Pigmented, raised	None	Lab. minus (L)	No FU
10	70	Biopsy	HSIL	Pigmented, plaque-like	None	Clitoris	VSK (19); VSK (23); thereafter NED (24)
11	33	Biopsy	None	None	HSIL	Lab. minus (L)	NED (84)
12	37	Biopsy	None	None	None	Mons pubis	NED (84)
13	48	Biopsy	None	None	Squamous hyperplasia	Lab. majus (R)	No FU

Pt.: patient; *age at diagnosis; y: years; H/O: History of; SCC: squamous cell carcinoma; dVIN: differentiated vulvar intraepithelial neoplasia; LS: lichen sclerosus; lab.: labium; NED: no evidence of (vulvar) disease; R: right; L: left; B/L: bilateral; FU: follow-up; HSIL: high-grade squamous intraepithelial lesion; CIN: cervical intraepithelial neoplasia; NILM: negative for intraepithelial lesion or malignancy

E4: Four (36%) of the 11 HPV-positive VSKs showed focal expression in upper epithelial layers (score = 1) [Table 3; Figure 3]. One HSIL showed extensive expression in the upper half of the epithelium (score = 2).

3.2.4 LCM-PCR

A total of 19 lesional areas and one area of adjacent normal epithelium were tested from the 11 HPV-positive VSKs. For 10 VSKs (14 lesional areas), the results of WTS-PCR could be validated on LCM-PCR [Table 4; Figure 3]. For 1 VSK, HPV could not be detected on LCM-PCR although 6 lesional areas were tested. This lesion was E4-positive, which signifies a productive HPV-infection. Therefore, for this lesion, E4-positivity was considered sufficient to rule out incidental detection of HPV from contamination. HPV was also not detected in the area of normal epithelium.

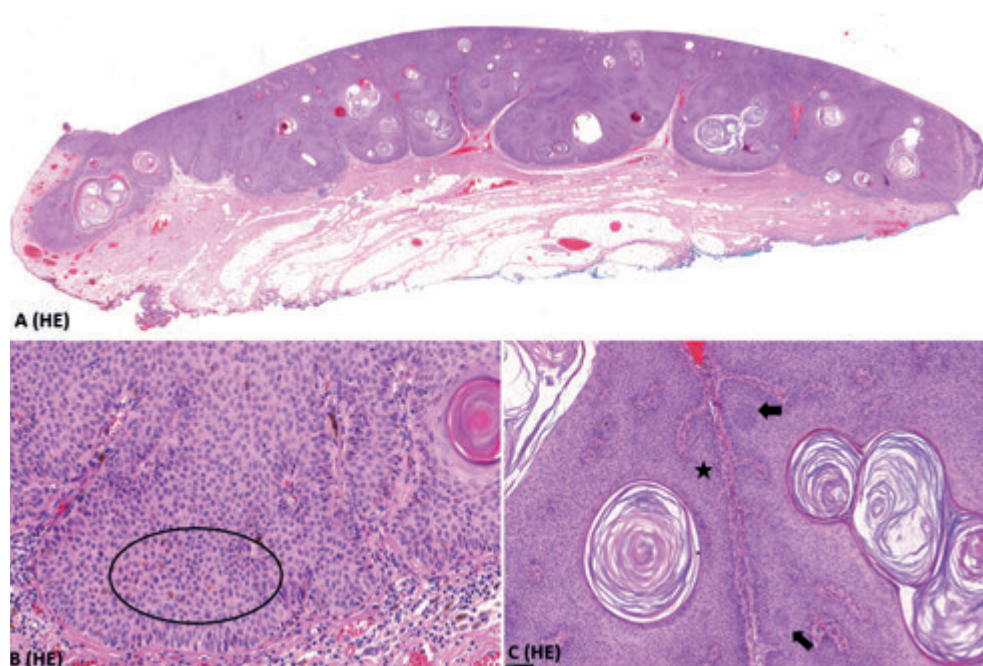


Figure 2: Histological appearance of the VSK from patient 9, in which HPV42 was detected on WTS-PCR (HE-stain); low (A) and high-magnification (B and C) images. A. Epithelial acanthosis and abundant horn pseudo-cysts can be appreciated (original magnification 2X). B. The lesion is composed of monomorphic basaloid cells with low nuclear-to-cytoplasmic ratio; circled area shows presence of melanin in the basaloid cells (original magnification 20X); C. Peripheral palisading (asterisk) and squamous eddies (arrows) are present within the lesion (original magnification 10X).

Table 3: Results of WTS-PCR, IHC, and LCM-PCR

Pt.	Age (y)	Lesions	Site	WTS-PCR (SPF10-LiPA25)	IHC		LCM-PCR	
					p16	E4 MIB-1		
1	67	VSK	Not specified	neg.	no expression	0 pos.	N/A	
2	53	VSK	Lab. majus	neg.	no expression	0 pos.	N/A	
3	66	VSK	Lab. majus	neg.	no expression	0 neg.	N/A	
4	52	VSK	Lab. majus	neg.	no expression	0 neg.	N/A	
5	46	VSK [‡]	Lab. minus	HPV6, 53	ext., patchy	1 pos.	HPV6, 53	
		HSIL (3 yrs before [‡])	Lab. majus	HPV16	block-type	0 pos.	N/A	
		Condyloma (1yr after [‡])	Lab. majus	HPV6	ext., patchy	0 pos.	N/A	
6	66	VSK	Lab. majus	HPV44	ext., patchy	1 pos. ¹	HPV44	
7	40	VSK	Lab. minus	HPV42	no expression	1 pos. ²	neg.	
8	25	VSK	Lab. majus	HPV6	ext., patchy	0 pos.	HPV6	
9	52	VSK	Lab. minus	HPV42	ext., patchy	1 pos.	HPV42	
10	70	VSK [§]	Clitoris	HPV44	ext., patchy	0 pos.	HPV44	
		HSIL (1 y. before [§])	Lab. minus	HPV16	block-positive	2 pos.	N/A	
		VSK (19 mons. after [§])	Clitoris	HPV44	no expression	0 pos.	HPV44	
		VSK (23 mons. after [§])	Lab. majus	HPV44	ext., patchy	0 pos.	HPV44	
11	33	VSK	Lab. minus	HPV6	ext., patchy	0 pos.	HPV6	
		HSIL (synchronous)	Lab. minus	HPV6, 16	block-positive	0 pos.	N/A	
12	37	VSK	Mons pubis	HPV untypable	ext., patchy	0 pos. ¹	HPV44*	
13	48	VSK	Lab. majus	HPV6	ext., patchy	0 pos.	HPV6	

Pt.: patient; y: years; WTS-PCR: whole tissue section; PCR: polymerase chain reaction; IHC: immunohistochemistry; neg.: negative; pos.: positive; N/A: not applicable; lab.: labium; ext.: extensive; HSIL: high grade squamous intraepithelial lesion; ¹few high power fields with nuclear staining in the upper 2/3rd of the epithelium; ²nuclear staining mostly in the upper 2/3rd of the epithelium; *HPV55 detected with the LiPA-25+ panel, which has been re-classified as HPV44

4. Discussion

4.1 Previous studies reported a higher prevalence of HPV in SKs compared to non-SK lesions of the FGT

There has been debate on whether the presence of HPV in SKs could be attributed to the incidental detection of resident HPV [6]. Our literature review provides interesting insights in this regard. We observed that some of the studies on SKs had also investigated the presence of HPV in non-SK genital lesions, and both groups of lesions were from women of comparable ages. Interestingly, in these studies, prevalence of HPV was much higher in SKs than in the non-SK lesions. If the detection of HPV in SKs was attributable to contamination, similar prevalence of HPV would be expected in both SKs and non-SK lesions.

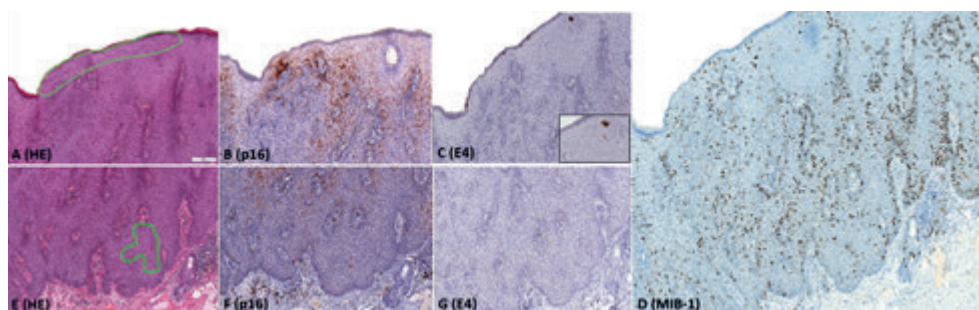


Figure 3: VSK from patient 9, in which HPV42 was detected on WTS-PCR. A. HE-stained lesional area; the area marked by the green line was analyzed using LCM-PCR and HPV42 was detected; B. p16-IHC: patchy expression; C. E4-IHC: focal expression in the superficial epithelial layer (score=1); inset shows the E4-positive cells under higher magnification; D. MIB-1-IHC: increased expression in the upper 2/3rd of the epithelial layer; E. HE-stained lesional area; the area marked by the green line was analyzed using LCM-PCR and HPV42 was detected; F. p16-IHC: patchy expression; G. E4-IHC is negative (white bar = 200µm).

There has also been debate on whether studies reporting on the presence of HPV in SKs used appropriate histological criteria for case inclusion. In their seminal article, Li and Ackerman opined that histological criteria used in some of these studies were insufficient for discriminating SKs from condylomas [4]. To address this issue, subsequent studies, including our current study, followed stringent histological criteria for case selection and excluded any lesions showing histological changes associated with active HPV-infection. Nevertheless, HPV was still detected in lesions that were judged as SK on histology [2,14]. Bai et al. and Tardio et al. therefore concluded that histology was not a reliable predictor of the HPV-status, and the results from our current series support this view.

Table 4: Results of LCM-PCR

Pt.	WTS-PCR (SPF10-LiPA25)	IHC-scores		LCM-PCR	
		p16	E4	LCM region	SPF 10-PCR
5	HPV6, 53	patchy	0	Lesional area – superficial / intermediate epithelial layer	HPV6
		patchy	1	Lesional area – superficial / intermediate epithelial layer	HPV6, 53
6	HPV44	patchy	0	Lesional area – basal / intermediate epithelial layer	HPV44
		neg.	0	Adjacent normal epithelium	neg.
7	HPV42	neg.	0	Lesional area – basal / intermediate epithelial layer	neg.
		neg.	0	Lesional area – intermediate epithelial layer	neg.
		neg.	1	Lesional area – superficial epithelial layer	neg.
		neg.	0	Lesional area – intermediate epithelial layer	neg.
		neg.	1	Lesional area – superficial / intermediate epithelial layer	neg.
		neg.	0	Lesional area – intermediate epithelial layer	neg.
		neg.	0	Lesional area – intermediate epithelial layer	neg.
8	HPV6	patchy	0	Lesional area – basal / intermediate epithelial layer	HPV6
9	HPV42	patchy	1	Lesional area – superficial epithelial layer	HPV42
		patchy	0	Lesional area – intermediate epithelial layer	HPV42
10 ^s	HPV44	patchy	0	Lesional area – basal / intermediate epithelial layer	HPV44
		neg.	0	Lesional area – basal / intermediate epithelial layer	HPV44
		patchy	0	Lesional area – basal / intermediate epithelial layer	HPV44
11	HPV6	patchy	0	Lesional area – basal / intermediate epithelial layer	HPV6
		patchy	0	Lesional area – basal / intermediate epithelial layer	HPV6
12	HPV untypable	patchy	0	Lesional area – basal / intermediate epithelial layer	HPV44*
13	HPV6	patchy	0	Lesional area – basal / intermediate epithelial layer	HPV6

Pt.: patient; LCM: Laser capture microdissection; PCR: polymerase chain reaction; WTS: whole tissue section; IHC: immunohistochemistry; ^sthree different lesions, *HPV55 detected with the LiPA-25+ panel, which has been re-classified as HPV44 neg.: negative

4.2 LR-HPV can produce lesions having the histology of VSK

In our series, HPV was detected in 73% (11/15) of the lesions having the histology of VSKs. The genotypes detected in these VSKs, i.e. LR-HPV6, LR-HPV44, LR-HPV42 and possibly-carcinogenic HPV53, have been previously detected in genital neoplasms in both sexes [2,3,14,15].

To augment HPV-testing, we performed IHC with the cellular biomarkers p16 and MIB-1, and the viral biomarker, E4. p16 is a tumor-suppressor protein that is overexpressed in HPV-infection as a consequence of cell-cycle deregulation by E6/E7 HPV-onco-proteins [16]. In HR-HPV-infection, the deregulation caused by E6/E7 is more pronounced, and this results in a block-type p16-expression [17]. In contrast, in transforming infections of LR-HPV, there is limited deregulation, and this may result in an extensive, non-block-type / patchy p16-expression. This pattern of p16-expression was observed in 82% (9/11) of the HPV-positive VSKs in our series [13,17]. However, patchy p16-expression, albeit, weak and focal, may also occur in normal vulvar tissue [17]. Currently, no reliable cut-offs have been identified that allows accurate distinction of the patchy p16-expression patterns of normal vulvar tissue from that of LR-HPV-infection, and this limits the usefulness of p16-IHC as a surrogate marker for LR-HPV-infection.

MIB-1 is a proliferation marker that is expressed predominantly in the parabasal layers in normal cervical or vulvar tissue. Pirog et al. observed that MIB-1 positivity, i.e. increased expression in the upper 2/3rd of the epithelium correlates strongly with the presence of HPV in vulvar lesions [11]. For VSKs, Bai et al. reported good concordance of MIB-1-expression and the HPV-status [15]. In our series, MIB-1 was positive in all the lesions (VSKs, HSILs, and condyloma) where HPV was detected on WTS-PCR. However, 2 (50%) of the HPV-negative VSKs also showed MIB-1 positivity. This suggests that MIB-1 has a higher sensitivity than specificity for predicting the presence of HPV in genital lesions.

The HPV-protein E4 helps disrupt the cytoskeletal structure of the squamous cells to allow viral release and transmission [16,18]. Therefore, accumulation of E4 in the upper layers of the epithelium indicates a productive infection [16,18]. E4-expression was seen in 4 (36%) of the HPV-positive VSKs in our series, and the expression pattern was similar to that previously reported for low-grade cervical intraepithelial neoplasia [13].

Using LCM-PCR, we further confirmed the presence of HPV in lesional cells expressing p16 and/or E4, as well as, in lesional cells in the basal/intermediate

epithelial layers, where HPV typically sets up infection. In contrast, an area of normal epithelium adjacent to the lesion was negative for HPV. These findings strongly argue against false-positivity due to surface contamination from resident HPV.

The latest edition of the WHO classification of FGT tumors suggests that HPV is more likely to be present in VSKs from younger women, than in those from older women [19]. In our series, however, 6 (55%) of the HPV-positive VSKs were from women > 40 years of age. Further investigations at a molecular level may help determine whether the HPV-positive VSKs, HPV-negative VSKs, and condylomas are a part of the same spectrum.

Our results suggest that LR-HPVs can produce lesions that are histologically identical to VSKs. Therefore, pathologists need to be aware that HPV-status of a lesion may not be accurately determined on histology alone. When diagnosing a lesion having the histology of SK, particularly in women with a history of HPV-related genital lesions, IHC with p16 and MIB-1 may be considered. Where p16 shows extensive, patchy expression, and MIB-1 shows increased expression in upper 2/3rd of the epithelium, a PCR may be performed to rule out HPV-infection.

4.3 Presence of LR-HPVs in VSKs may influence the clinical management

HPV-infection is known to generate a ‘field-effect’, which predisposes to co-infection with other LR/HR-HPV genotypes [7]. This may result in synchronous or metachronous lesions in contiguous anatomical sites. In our series, 4 patients with HPV-positive VSKs had a previous or co-existent vulvar HSIL. Therefore, thorough examination of the genital skin and follow-up can be important for patients with HPV-positive lesions.

LR-HPV-infection has also been reported to unbalance the vaginal microbiota by facilitating over-proliferation of anaerobic bacteria, such as *Gardnerella* [20]. Therefore, to avoid a bacterial or fungal co-infection, treatment strategies for HPV-positive VSKs should be formulated to address the viral cause.

Determining the prevalence of HPV in lesions having the histology of VSKs will also help to correctly estimate the burden of LR-HPV-related disease, which is vital for informing and updating preventive strategies, such as HPV-vaccination.

4.4 Strengths and limitations

We provide a comprehensive review of the literature on a contentious area in diagnostic pathology. To the best of our knowledge, this is the first study to investigate the association of HPV with lesions having the histology of VSKs, using both WTS-PCR and LCM-PCR. The sample size of this study was small, but was comparable to previous studies and is attributable to the rarity of the disease. For HPV-detection, we used the analytically sensitive SPF10-DEIA-LiPA25-system, which can detect HPV-DNA optimally from FFPE-tissues, despite the formalin-induced degradation of the genetic material. Due to the higher sensitivity, DNA-PCR is preferred over DNA-in-situ hybridization (ISH) for detecting HPV from benign lesions having a low viral load [21]. The novel RNA-ISH-based RNAscope® may offer a higher sensitivity than DNA-ISH, however, further research is required to delineate cut-offs for signal interpretation in lesions with low HPV-transcript numbers [22].

Our study, however, may suffer from a selection bias, as some of the patients in our series were under follow-up for other vulvar diseases. This limits the generalizability of our results. Furthermore, due to the retrospective nature of our study, paired normal vulvar tissue could not be tested as controls, and this is another limitation.

5. Conclusion

Our results, based on the literature review and our case-series, suggest that LR-HPV-infection can produce lesions in the FGT that have the histology of SKs and do not exhibit features associated with HPV-infection. While diagnosing lesions having the histology of SKs, a potential association with HPV should be borne in mind by pathologists, even for SKs from older women. To facilitate appropriate management, HPV-testing should be performed for lesions having the histology of SKs, particularly for women with previous or concurrent HPV-associated genital lesions.

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Supplementary materials can be downloaded from this link: <https://drive.google.com/drive/folders/1tL1EPC2TEEPskCemSyHDZ7t30sYKZnyk?usp=sharing>

3



CHAPTER 3

PRECURSOR LESIONS OF VULVAR SQUAMOUS CELL CARCINOMA – HISTOLOGY AND BIOMARKERS: A SYSTEMATIC REVIEW

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Abstract

The precursor lesion of vulvar squamous cell carcinoma (VSCC), namely vulvar intraepithelial neoplasia (VIN), is classified as: human papillomavirus (HPV)-related high grade squamous intraepithelial lesion (HSIL), and HPV-independent differentiated VIN (dVIN). Traditionally, histology and immunohistochemistry (IHC) have been the basis of diagnosis and classification of VIN. HSIL shows conspicuous histological atypia, and positivity on p16-IHC, whereas dVIN shows less obvious histological atypia, and overexpression or null-pattern on p53-IHC. For both types of VIN, other diagnostic immunohistochemical markers have also been evaluated. Molecular characterization of VIN has been attempted in few recent studies, and novel genotypic subtypes of HPV-independent VSCC and VIN have been identified.

This systematic review appraises the VSCC precursors identified so far, focusing on histology and biomarkers (immunohistochemical and molecular). To gain further insights into the carcinogenesis and to identify additional potential biomarkers, gene expression omnibus (GEO) datasets on VSCC were analyzed; the results are presented.

1. Introduction

Vulvar squamous cell carcinoma (VSCC) constitutes 90% of all vulvar malignancies, and arises from the precursor lesion, vulvar intraepithelial neoplasia (VIN) [1]. Around 1/3rd of VSCC are caused by human papillomavirus (HPV), and the precursor lesion for this group is usual VIN/high grade squamous intraepithelial lesion (uVIN/HSIL) [1, 2]. The majority of VSCC are, however, HPV-independent and arise on the background of chronic dermatoses. Somatic mutations of *TP53* have been implicated in the pathogenesis of this category, and the precursor lesion is called differentiated VIN (dVIN) [1, 2].

Traditionally, histology and immunohistochemistry (IHC) have been the basis of diagnosis and classification of VIN and VSCC. In recent years, for several cancers, advanced molecular analyses have allowed more objective classification, and identification of diagnostic and prognostic biomarkers. For VIN and VSCC, molecular characterization has been attempted in a limited number of studies so far.

This systematic review was performed with the objective of appraising the histological features of, and the biomarkers (immunohistochemical and molecular) for VSCC precursors. To gain further insights into VSCC carcinogenesis, publicly available datasets on expression profiling of VSCC were analyzed; these results are also presented.

2. Materials and methods

2.1 Literature Review

The recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) protocol were followed [3]. The review was registered in the International Prospective Register of Systematic Reviews (PROSPERO), with accession number CRD42019107290 [4].

Electronic search strategies combining Medical Subject Headings (MeSH) and free-text words were prepared, with the help of medical librarians at Erasmus MC. Biomedical bibliographic databases, viz. Embase.com, MEDLINE Epub (Ovid), Cochrane Central Register for Controlled Trials (CENTRAL), Web of Science (Science and Social Science Citation Index), were searched. The full search strategy is provided in supplementary document 1. Date restriction was

not applied. The last search was conducted in May, 2019. Citation, reference, and hand searching were additionally performed.

Original articles and review articles describing either the histology, or immunohistochemical or molecular markers of VSCC precursors, and written in English were included. Case reports, conference abstracts, animal studies, and in-vitro studies were excluded.

A total of 1112 references were retrieved, of which 373 were included after the first round of screening by one author (SDG). These 373 references were independently screened by three other authors (PEG, SK, and FvK) by reading the titles. PEG, SK, and FvK are practising pathologists with substantial experience in the subject content. One hundred and fifty four references met consensus for inclusion; full text was available for 127 of these. Nine additional references were identified through reference searches. The final inclusion constituted 106 articles (99 original articles, 7 reviews). The process of reference selection is delineated in Figure 1. Due to variability in the nomenclature, and heterogeneity in the published data, a meta-analysis could not be performed, and a narrative synthesis was prepared.

2.2 Gene Expression Omnibus (GEO) DataSet analysis

Datasets on gene expression analyses of VSCC were searched on GEO. Four datasets were identified, of which 3 were discarded; 2 owing to very low number of samples, and 1 due to lack of intensity values. The included dataset (GSE38228) contained gene expression data from 14 VSCC and 5 normal vulvar samples, performed on Illumina Human HT-12 V4.0 [5]. The signal intensity data had been 2log transformed (base2) and quantile normalised [6].

The data was imported into OmniViz (version 6.1.13.0, BioWisdom Ltd., Cambridge, UK) for further analysis. For each probe set, the geometric mean of the hybridization intensity of all samples was calculated, and the level of expression was assessed relative to this geometric mean and 2log-transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes between VSCC and controls were identified using statistical analysis of microarrays (SAM). Cutoff values for significantly expressed genes were a false discovery rate (FDR) of ≤ 0.01 , and a fold change of 1.5. Functional annotation of the SAM results was done using

Ingenuity Pathway Analysis (IPA, QIAGEN, Mountain View, CA), and Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8, NIAID/NIH).

3. Results

The information extracted from the literature, and the results of GEO analyses were categorised as pertaining to HPV-related VSCC precursors, or HPV-independent VSCC precursors, and are presented in the subsequent sections. The evolution of terminology, etiopathogenesis, and clinical features for both categories, extracted from the included literature, are also briefly discussed.

3.1 *Evolution of terminology*

The term intraepithelial neoplasia was introduced in 1967, and was adopted by the International Society for the Study of VulvoVaginal Diseases (ISSVD) in 1986 [7, 8]. Initially, a three-tier grading system for VIN (VIN I, II, III) was recommended. The VIN I category was later removed, as this almost never progressed to VSCC [9]. In 2004, the ISSVD scheme was modified to include usual VIN (uVIN), and differentiated VIN (dVIN), to signify HPV-related and HPV-independent VSCC precursors respectively [9].

With a view to achieving uniformity in terminology, in 2012, the Lower Anogenital Squamous Terminology (LAST) committee recommended the terminology of squamous intraepithelial lesion (SIL) for VSCC precursors, classified as low grade SIL (LSIL) and high grade SIL (HSIL) [10]. The 2014 World Health Organization (WHO), and 2015 ISSVD classifications accepted the SIL terminology for HPV-related VIN, and retained dVIN as a separate category [1, 2]. In this review, we used HSIL, and dVIN to refer to the precursors of HPV-related VSCC and HPV-independent VSCC respectively.

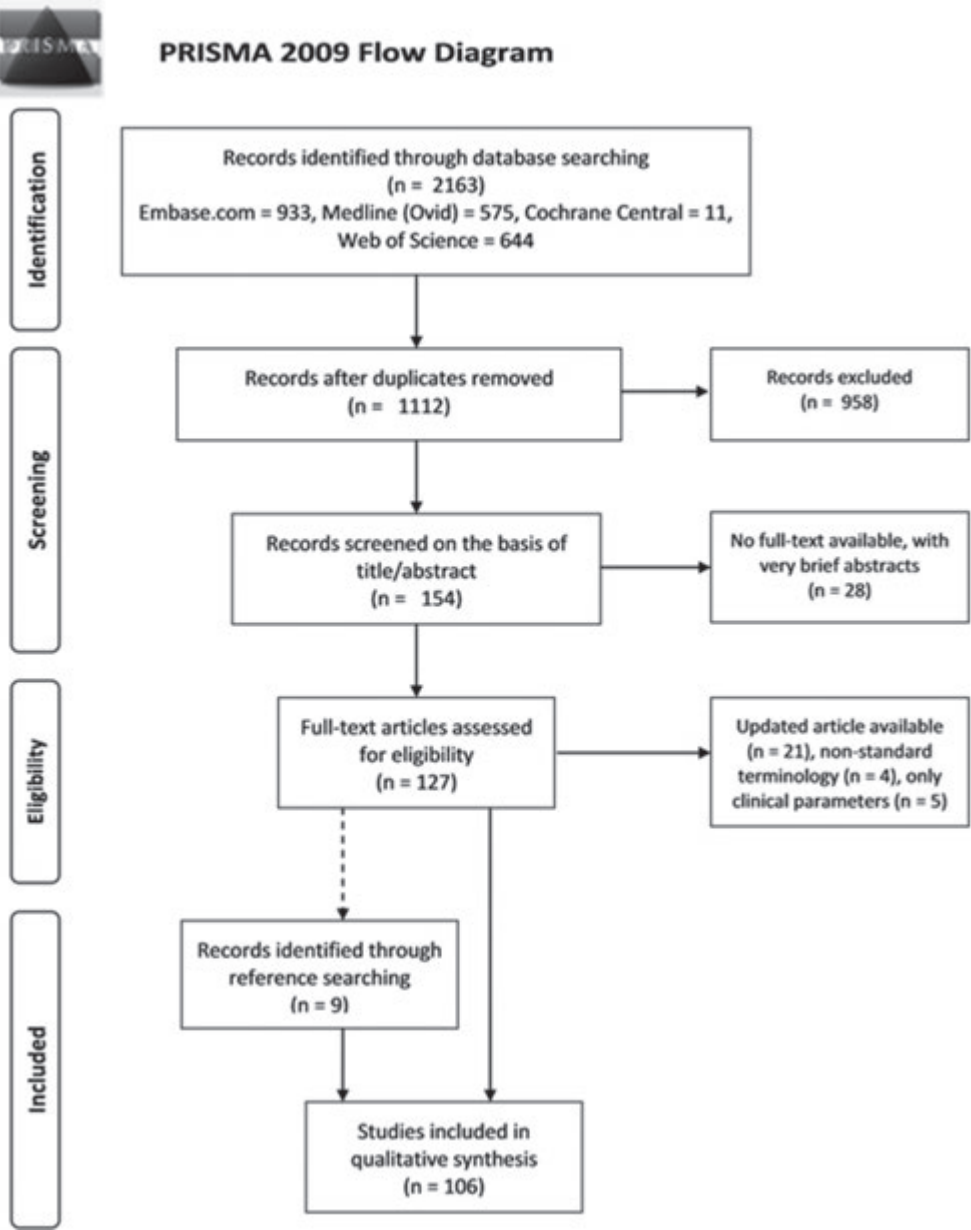


Figure 1: PRISMA flow diagram depicting the process of reference selection

3.2 HPV-related VSCC precursors (HSIL)

3.2.1 Etiopathogenesis

More than 80% of HSIL have been reported to be HPV-positive, frequently detected genotypes being high-risk HPV16 (77.2%), HPV33 (10.6%), and HPV18 (2.6%) [11-19].

Similar high rates of HPV-positivity, however, have not been found in VSCC. Only 28.6% of cases from an international cohort of 1709 VSCC were found to harbor HPV, in 75% of which, HPV16 was detected [20]. Other studies have reported 15 – 79% HPV-positivity in VSCC [21]. Low-risk HPV6 and HPV11 have been infrequently reported in VSCC; their exact role in the carcinogenesis is unclear [22, 23].

High-risk HPV mediates carcinogenesis primarily through the oncoproteins E6 and E7; these interfere with the functioning of tumor suppressor retinoblastoma protein (pRB), and p53 [24]. Loss of pRB generates oncogenic stress, potentially leading to p16INK4a over-expression, and thus allowing hyper-proliferation of the infected cells [24, 25].

To facilitate carcinogenesis, persistent HPV-infection also induces a local immunosuppressive microenvironment, characterized by reduced concentrations of (CD1a+ and CD207+) dendritic cells and HPV-specific (CD4+ and CD8+) T-cells, and upregulation of T-regulatory cells [26-28]. Treatment with imiquimod is known to normalize (CD4+, CD8+) T-cell counts in the epidermis, and bring about viral clearance [28].

3.2.2 Clinical features

HSIL most commonly affects women of 40 – 44 years, with a second peak after 55 years [14, 20, 28, 29]. Patients commonly present with pain, pruritus, or dysuria, although 40% can be asymptomatic [30]. Clinically, these lesions can be whitish, erythematous, or pigmented macules, papules, or verrucous plaques [14, 31-33]. HSIL can be multifocal in 70% of cases, and the commonest locations are the labia minora and perineum [30-33]. Due to the ‘field effect’ induced by HPV, patients with HSIL may have concurrent involvement of other ano-genital sites [27, 29, 31-35]. A thorough examination of the entire lower genital tract is therefore recommended for all HSIL patients.

HSIL has a relatively low risk of progression to VSCC: 9 – 16% for untreated cases, and 3% for treated cases. Around 1.2% of HSIL spontaneously regress [29, 31, 34, 36]. The reported recurrence rate for HSIL varies between 13 – 36% [27, 31, 36, 37].

3.2.3 Histological features

Histological features of HSIL were extracted from 9 studies [27, 38-45]. The architectural and cytological abnormalities of HSIL are conspicuous, and often apparent under low magnification [Figure 2]. A basophilic (basaloid) appearance can frequently be appreciated in HSIL [44]. The key identifying features include high nuclear-to-cytoplasmic ratio, hyperchromasia, pleomorphism, multinucleated cells, mitoses, and apoptotic bodies [Figure 2] [43]. The disturbed orientation of the dysplastic epithelial cells often render a ‘wind-blown’ appearance to HSIL [43]. Tangential sectioning of HSIL with extensive down-growths, or with involvement of skin appendages, can mimic early invasion and these should be interpreted with caution [39-41]. The histological features are listed in Table 1.

Two histological variants of HSIL have been reported: warty/condylomatous, and basaloid/undifferentiated [43]. Warty HSIL has an acanthotic or papillary surface, with deep and wide rete ridges, abundant koilocytes, and dyskeratotic cells [42, 43]. Basaloid HSIL is flat, with small, uniform, basaloid cells often replacing the entire epithelium [42, 43]. Since some HSIL exhibit histological features from both variants, it has been assumed that these are a part of the same spectrum.

HSIL can also histologically mimic dVIN, with a pooled prevalence of 2% [44-46]. Similarly to dVIN, these show cytoplasmic eosinophilia due to abnormal keratinization, spongiosis, and atypia limited to the basal and parabasal layers [45]. Rakislova et al. demonstrated p16-positivity, and detected HPV16 in these dVIN-like HSIL [45]. HSIL with superimposed lichen simplex chronicus (LSC) can also mimic dVIN histologically, but can be correctly diagnosed through p16-positivity on IHC [47]

3.2.4 Immunohistochemical markers

Immunohistochemical markers studied in HSIL are presented below, and summarized in Table 2. This information was extracted from 24 studies [24, 25, 35, 45, 47-66]. The immunohistochemical markers have been categorized as per their sub-cellular location as nuclear, cytoplasmic, or extra-cellular. The biological processes and canonical pathways associated with these markers are presented in Table S1 (supplementary document 2).

A. Nuclear markers

(i) p16: As per LAST guidelines, continuous/diffuse/band-like/block-like strong nuclear, or nuclear with cytoplasmic p16-expression in the basal layer, extending up to at least one-third of the epithelial thickness, is to be interpreted as 'positive' p16-staining [Figure 3] [10]. Cytoplasmic staining alone, or patchy focal staining without staining in the basal layers should be interpreted as p16-negative. Positivity with p16-IHC has been reported for stand-alone HSIL, as well as HSIL adjacent to VSCC [24, 45, 47-52]. With p16-IHC, improvement in the inter-observer agreement in HSIL diagnosis has been reported [10].

The combination of positive HPV-polymerase chain reaction (HPV-PCR), and block-positive p16-IHC is considered to be the 'gold standard' for diagnosing HSIL [44]. However, p16-IHC alone is considered a reliable surrogate marker for HPV-infection [25, 48-51]. p16-IHC can also help distinguish HSIL from benign mimics, such as transition zone mucosa, squamous hyperplasia, or radiation change, which are p16-negative [10].

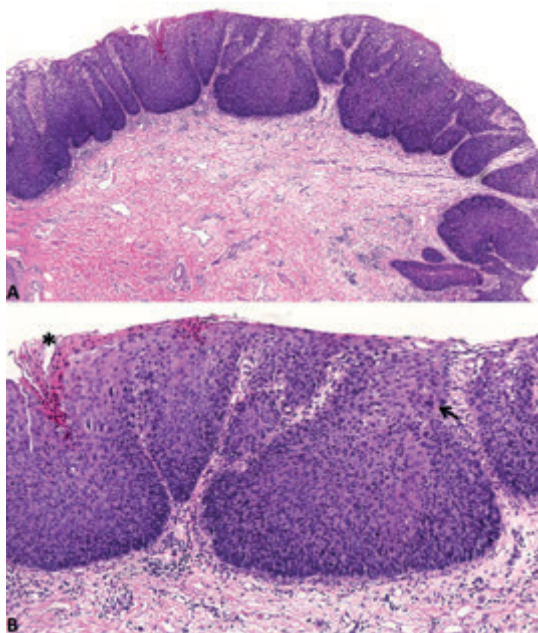


Figure 2: High grade squamous intraepithelial lesion (HSIL) with characteristic histological features; hematoxylin-eosin (HE) stain. A. Acanthosis with club shaped rete ridges, and a basophilic appearance of the epithelium can be appreciated under low magnification (original magnification 25X); B. Cellular crowding, anisonucleosis, pleomorphic cells with high nuclear cytoplasmic ratio (arrow), hyperkeratosis and parakeratosis (asterisk) can be observed under higher magnification (original magnification 100X).

(ii) p53: On p53-IHC, HSIL usually shows wild-type (wt) staining, characterized by sporadic nuclear staining, with weakly positive to completely negative basal epithelial layer [Figure 3]. In some HSIL, 'accentuated wt-p53' pattern is seen, characterized by weak, patchy staining in the basal layer, and a higher proportion of positive nuclei in the suprabasal layers [52]

(iii) Cyclin-D1 and pRB: Loss of pRB-expression in HSIL and VSCC reflects disruption of pRB by HPV. The cyclinD1-CDK4 complex reverses the tumor suppressor effect of pRB, and allows cell-cycle progression. Normal vulvar epithelium is negative for cyclin-D1, whereas its over-expression in both the nucleus and cytoplasm has been reported in both HSIL and VSCC [24, 53].



Figure 3: High grade squamous intraepithelial lesion (HSIL), histology and p16 and p53 immunohistochemistry (IHC), original magnification 50X. A. Hematoxylin-Eosin (HE) stained section shows the characteristic histology of HSIL; B. Block-like positivity can be appreciated on p16-IHC; C. On p53-IHC, scattered nuclear staining in the suprabasal layers, with lack of staining in the basal layers is seen.

(iv) Histone deacetylases-class I (HDAC1): HDACs represses transcription by increasing the affinity of histone complexes for DNA, and also modifies the transcription factors p53, E2F, and pRB. Increased nuclear expression of HDAC1,2, and 3 have been reported for HSIL and VSCC [54].

(v) E-Cadherin (CDH1)/ β -Catenin (CTNNB1): E-cadherin/ β -catenin complex regulates cellular adhesion, proliferation, and survival. Dysfunction of this complex has been implicated in carcinogenesis [55]. In non-neoplastic vulvar lesions, CDH1 and β -catenin are strongly expressed in the epithelial cell membranes [55]. In HSIL, expression of both in the cytoplasm, with or without expression in the cell membrane is seen [55].

Table 1: Histological features of HSIL, and dVIN

Histological features of HSIL		
<i>Knight [38]</i>	<i>Other literature [27, 38-45]</i>	<i>WHO 2014 criteria [1]</i>
1. Hyperkeratosis	1. High nuclear-cytoplasmic ratio	1. Epithelial cell hyperchromasia
2. Parakeratosis	2. Hyperchromasia	2. Cellular crowding
3. Acanthosis with club shaped rete ridges	3. Pleomorphism	3. Anisonucleosis
4. Disorientation of the individual cells commencing above the basal cell layer with variable extension to the surface	4. Multinucleated cells	4. Acanthosis
5. Nuclear clumping with mitotic figures	5. Mitoses	5. Parakeratosis
6. Intact basement membrane	6. Apoptotic bodies	6. Hyperkeratosis
		7. Variable HPV cytopathic effect
Histological features of dVIN		
<i>Yang and Hart [48]</i>	<i>Other literature [27, 41, 43, 44, 86, 94-98]</i>	<i>HO 2014 criteria [1]</i>
1. Variable nuclear atypia, from slight-to-moderate degree, to occasionally severe	1. Basal cellular atypia (including disarray of the basal cellular layers, large pleomorphic keratinocytes)	1. Basal cell atypia, with nuclear hyperchromasia
2. Hyperchromatic nuclei with irregular contours in the basilar cells	2. Angulated nuclei	2. Karyomegaly
3. Vesicular nuclei	3. Atypical mitosis in the basal layer	3. Prominent nucleoli
4. Macronucleoli	4. Mitotic count > 5 / 5mm	4. Atypical mitoses in the basal layer
5. Binucleated cells	5. Prominent nucleoli/ Macronucleoli	5. Dyskeratosis
6. Scattered mitoses, in basilar and suprabasilar layers	6. Individual cell keratinisation	6. Individual cell keratosis
7. Enlarged cells	7. Deep keratinisation	7. Elongation and anastomosis of rete ridges
8. Elongated and frequently branched rete ridges	8. Deep squamous eddies	
9. Abundant, brightly eosinophilic cytoplasm	9. Cobblestone appearance	
10. Whorls of differentiated cells, with/without keratin pearls	10. Hypermaturation of rete ridges	
11. Prominent intercellular bridge	11. Elongation and anastomosis of rete ridges	
	12. Hyperplasia/Acanthosis	
	13. Hyperkeratosis	
	14. Parakeratosis	

(vi) SRY-box 2 (SOX2): SOX2 controls pluripotency in both embryonic and adult tissue-specific stem cells, and can induce pluripotency in adult somatic cells [56]. Weak nuclear SOX2-expression in the basal and parabasal epithelial layers is seen in normal vulva. Increased, strong nuclear SOX2-expression, particularly in the middle and upper-third of the epithelium, has been reported for HSIL [56].

(vii) H2A histone family member X (γ -H2AX): γ -H2AX is generated from phosphorylation of the histone protein H2AX, in response to DNA-double strand breaks. γ -H2AX induces chromatin modification, and apoptosis [57]. Diffuse or granular nuclear staining with γ -H2AX in all the epithelial layers has been reported for HSIL [57].

(viii) GATA-binding protein 3 (GATA3): GATA3 is known to upregulate the MDM2-proto-oncogene, which in turn downregulates and degrades p53. Moderate to strong nuclear GATA3-expression in non-neoplastic vulva, as well as in HSIL has been reported [58].

Table 2: Immunohistochemical markers that have been studied for HSIL, and dVIN

Biomarker	Lesion(s) studied	Location	Molecular Functions*
p16 (CDKN2A) [10, 24, 25, 45 – 52]	HSIL, dVIN	nucleus	transcription regulator; DNA binding; NF- κ B binding; p53 binding; protein kinase binding; RNA binding; ubiquitin-protein ligase inhibitor activity
p53 [48, 52, 60, 99-103]	HSIL, dVIN	nucleus	transcription regulator; DNA binding; histone deacetylase regulator activity; protein heterodimerization/homodimerization activity
CCND1 [24, 53]	HSIL, dVIN	nucleus	transcription regulator; histone deacetylase binding; kinase activity
pRB [24, 53]	HSIL	nucleus	sequence-specific DNA binding; transcription factor; ubiquitin protein ligase binding
Ki-67/MIB-1 [35, 51]	HSIL, dVIN	nucleus	ATP binding; nucleotide binding; protein binding
HDAC1 [54]	HSIL	nucleus	transcription regulator; NF-kappaB binding; p53 binding
CTNNB1 [55]	HSIL, dVIN	nucleus	transcription regulator; cadherin binding; chromatin binding; SMAD binding
CDH1 [55]	HSIL**	plasma membrane	alpha-catenin binding; beta-catenin binding; cell adhesion molecule binding; cytoskeletal protein binding; gamma-catenin binding
SOX2 [56]	HSIL, dVIN	nucleus	transcription regulator
γ -H2AX [57]	HSIL	nucleus	transcription regulator

Biomarker	Lesion(s) studied	Location	Molecular Functions*
Survivin [57, 60]	HSIL	cytoplasm	chaperone binding; cofactor binding; cysteine-type endopeptidase inhibitor activity involved in apoptotic process; enzyme binding; protein heterodimerization / homodimerization activity; Ran GTPase binding; tubulin binding
GATA3 [58]	HSIL, dVIN	nucleus	transcription regulator; HMG box domain binding; interleukin-2 receptor binding
MCM2 [59]	HSIL	nucleus	enzyme - 3'-5' DNA helicase activity; ATP binding; histone binding
TOP2A [59]	HSIL	nucleus	enzyme - ATPase activity; histone deacetylase binding; ubiquitin binding
hTERT [60]	HSIL	nucleus	enzyme - nucleotidyltransferase activity; protein binding; tRNA binding
p-S6 [61]	HSIL, dVIN	nucleus	mRNA binding; protein binding; structural constituent of ribosome
STMN1 [62]	HSIL	cytoplasm	protein binding; tubulin binding
FSCN1 [63]	HSIL, dVIN	cytoplasm	actin binding; cadherin binding; RNA binding
CK17 [64, 96]	HSIL, dVIN	cytoplasm	MHC class II protein binding / receptor activity; structural constituent of cytoskeleton
AKT1 [65]	HSIL	cytoplasm	enzyme - kinase activity, transferase activity; calmodulin binding
MMP2 [66]	HSIL	extracellular space	enzyme - hydrolase activity, metalloendopeptidase activity
CK13 [96]	dVIN	cytoplasm	protein binding; structural molecule activity
ER [105]	dVIN	nucleus	ligand-dependent receptor; DNA binding; transcription factor binding
PR [105]	dVIN	nucleus	ligand-dependent receptor; DNA binding

*From IPA Gene View (QIAGEN); **This marker was studied in dVIN, but the pattern of expression was same as that in the normal vulvar epithelium

(ix) ProEx C: ProEx C is an antibody cocktail targeting the nuclear enzymes minichromosome maintenance complex component 2 (MCM2), and topoisomerase IIa (TOP2A). MCM2 influences mitotic G1/S phase transition. TOP2A modulates chromosome condensation and segregation, and is activated in response to genotoxic stress. For HSIL, diffuse nuclear staining with ProEx C, from lower one-third to full epithelial thickness, has been reported. In normal vulvar epithelium, ProEx C only stains the basal and parabasal layers [59].

(x) Human Telomerase Reverse Transcriptase (hTERT): hTERT extends and maintains telomeres, and enables cells to overcome replicative senescence. In HSIL, nuclear staining with hTERT follows the distribution of atypical keratinocytes,

being limited to < 50% of the epithelial cells in most cases. hTERT staining only in the basal/parabasal layers has been noted in normal vulvar epithelium [60].

(xi) Ki-67/MIB-1: Ki-67 is a nuclear antigen present in proliferating human cells in all stages of the cell-cycle, except the G0 phase [35, 51]. MIB-1 is the monoclonal antibody against Ki-67. In normal vulvar epithelium, MIB-1 stains mainly the parabasal layers, and infrequently the basal layers [51]. In HSIL, increased MIB-1 staining in both basal and parabasal layers, extending into the upper two-thirds of the epithelium can be seen [35, 51].

(xii) Phosphorylated-S6 (p-S6): Hyperactivity of the EGFR/ERK, and PI3K/AKT/mTOR signaling pathways, which are involved in SCC, leads to phosphorylation of the ribosomal protein S6, which modulates mRNA translation [61]. In normal vulvar epithelium, the basal and suprabasal cells are negative for p-S6, whereas in HSIL, the basal cells show nuclear p-S6-expression [61].

B. Cytoplasmic markers

(i) Stathmin (STMN1): STMN1 is a microtubule-destabilising phosphoprotein, which regulates mitosis [62]. Normal vulvar mucosa does not express STMN1, whereas in HSIL, cytoplasmic expression in more than one-third of the epithelial thickness has been reported [62].

(ii) Fascin-1 (FSCN1): FSCN1, an actin-bundling protein, promotes migration and invasion of carcinoma cells. Increased cytoplasmic FSCN1-expression in all epithelial layers, except in the surface parakeratotic cells, has been reported for HSIL [63]. FSCN1 immunoreactivity is limited to the lower-third of the epithelium in normal vulva.

(iii) Cytokeratin 17 (CK17): The intermediate filament protein CK17, regulates protein synthesis and cell growth, and is expressed in activated keratinocytes in the suprabasal layers of epidermis. In HSIL, focal cytoplasmic CK17-staining of weak or moderate intensity, in the superficial epithelial layers, has been reported [64].

(iv) Survivin: Apoptosis-inhibitor protein survivin is overexpressed in human cancers, and is considered an unfavorable prognostic marker [57, 60]. In normal vulva, survivin stains the cytoplasm of parabasal/basal epithelial cells, whereas in HSIL, the staining pattern has been reported to follow the extension of the atypical cells within the epithelium [60].

(v) AKT1: AKT1 is a serine/threonine kinase, down-regulated by cutaneous HPV-types to weaken the keratin envelope and allow viral release [65]. AKT1 loss in HSIL correlates with high copy numbers of episomal HPV16, i.e. is indicative of early HPV-infection. Expression of AKT1 in HSIL correlates with low copy numbers of episomal HPV16, and indicates HPV-integration [65].

C. Extracellular markers

Matrix metalloproteinase (MMP2): MMP2, an enzyme of the metalloproteinase family, degrades type IV collagen and fibronectin in the basement membrane, and facilitates stromal and vascular invasion by tumor cells [66]. Granular or diffuse cytoplasmic staining with MMP-2 in stromal cells has been reported in HSIL and VSCC [66].

3.2.5 Molecular Markers

A. Allelic imbalances, loss of heterozygosity (LOH), copy number alterations (CNA)

Loss of chromosome (chr) 3p and gain of chr 3q has been most frequently reported in HSIL and VSCC [67-75]. LOH at chr 2.4 and chr 8.2 has been identified in both HPV-related VSCC, and the adjacent HSIL [67]. Single studies have reported loss of chr 13q, and gain of chr 20p and chr 20q for both HSIL and VSCC [68, 72]. In addition, gain of chr 8q, and loss of chr 8p, chr 11, and chr 17 in VSCC have been reported [68-72, 74].

For HSIL that progressed to VSCC, Swarts et al. identified chr 1p, 1q, 3q, 20 gains and chr 4 loss [76]. Interestingly, in VSCC samples with an adjacent HSIL, chr 1pq gains could be detected only in the HSIL, and not in the VSCC, potentially implying intratumoral heterogeneity [76]. Swarts et al. also observed distinct patterns of CNA in HSIL and dVIN, and that CNA were more frequent in HSIL than in dVIN [76].

B. Somatic mutations

Nooij et al. detected *TP53* mutations in both HSIL and VSCC, albeit in much lower frequency than in HPV-independent counterparts [77]. Contrasting findings were reported by Zięba et al., who detected *TP53* mutations with comparable frequency in HPV-related, and HPV-independent VSCC [78]. They ascribed this difference to sequencing techniques and the highly sensitive HPV-detection method used [78].

HRAS mutations have been detected in both HSIL and VSCC (0 – 14%), whereas *NOTCH1* mutations have been detected only in HSIL [77, 79]. Other mutations that have been detected in HPV-related VSCC include *PIK3CA* (7 – 33%), *CDKN2A* (0 – 25%), *PTEN2* (9%), *FGFR3* (14%), *KIT* (18%), *BRCA2* (17%), *FBXW7* (3 – 17%) [77-80].

C. Microsatellite instability (MSI)

Only a single study has reported MSI for HPV-related VSCC [67].

D. Epigenetic changes

Hypermethylation of *CDKN2A* (9.1 – 15.4%), stratifin (45.5 – 53.8%), *TSR1* (20%), and *TSLC1* (44.4%), has been reported for HSIL and VSCC, using methylation-specific PCR (ms-PCR) [81-83].

E. Expression profiling

Upregulation of genes involved in cell-cycle regulation and proliferation, with the exception of *cyclins D1* and *D2*, has been reported for HSIL [84]. *Cyclins D1* and *D2* were significantly downregulated, probably due to the pronounced upregulation of *CDKN2A* [84]. Upregulation of Fanconi genes (*FANCA*, *FANCD2*, and *FANCE*), and *BRCA1* have been reported in HSIL, which probably reflects the response to the HPV-induced DNA damage. Downregulation of nuclear androgen and estrogen receptors (*AR* and *ESR1*) potentially indicates reduced paracrine and endocrine regulation [84]. Cell-cell adhesion molecules (*ASAM*, *SLIT2*, *ITGA8*, *FN1*, *EPDR1*) were also downregulated in HSIL, indicating its tendency towards invasion [84, 85].

3.2.6 GEO DataSet analysis

Based on the expression levels of p16 (*CDKN2A*), 3 samples were identified as HPV-related VSCC. In these samples, expression of 1117 probe sets (675 up and 442 down) differed significantly from the controls. Of these, 342 probe sets (244 up and 98 down) were found to be exclusively involved in HPV-related VSCC, i.e. not involved in HPV-independent VSCC. The associated biological processes, canonical pathways, and the upstream regulators of these differentially expressed genes are discussed below.

A. Biological Processes

The associated biological processes were identified through Gene Ontology (GO) analysis using DAVID. Those with the most significant p-values are depicted in Figure 4. These included processes related to host immune response to HPV-infection, organismal injury and death, and proteolytic activity mediated by proteasomes through ubiquitin-ligation.

B. Pathways

Based on the $-\log(p\text{-values})$ of the differentially expressed genes, the associated canonical pathways were identified using IPA, and are depicted in Figure 5. The protein ubiquitination pathway was most significantly upregulated. This signaling pathway helps degrade regulatory proteins involved in cell proliferation, apoptosis, DNA repair, and antigen presentation. Several cancers are known to manipulate the ubiquitin pathways to ensure tumor cell survival and metastasis. Other significantly upregulated pathways i.e. antigen presentation pathway, interferon signaling, and phagosome maturation, regulate cellular immune response. The systemic lupus erythematosus (SLE)-signaling pathway mediates aberrant activation of T-cells, and is known to play a role in cellular growth and proliferation.

C. Upstream regulators

Based on the z-scores, 95 upstream regulators (74 activated, 21 inhibited) were identified using IPA [Table S2, supplementary document 2]. These comprised protein complexes, cytokines, enzymes, G-protein coupled receptors, transmembrane receptors, growth factors, ligand-dependent nuclear receptors, miRNAs, transcription and translation regulators, and transporters. The top five activated upstream regulators were the cytokines, tumor necrosis factor (TNF), interferons gamma and alpha2 (IFNG, IFNA2) and prolactin (PRL), and the transcription regulator, interferon regulatory factor 7 (IRF7). All of these are primarily associated with pro-inflammatory functions, and host-response to viral infections.

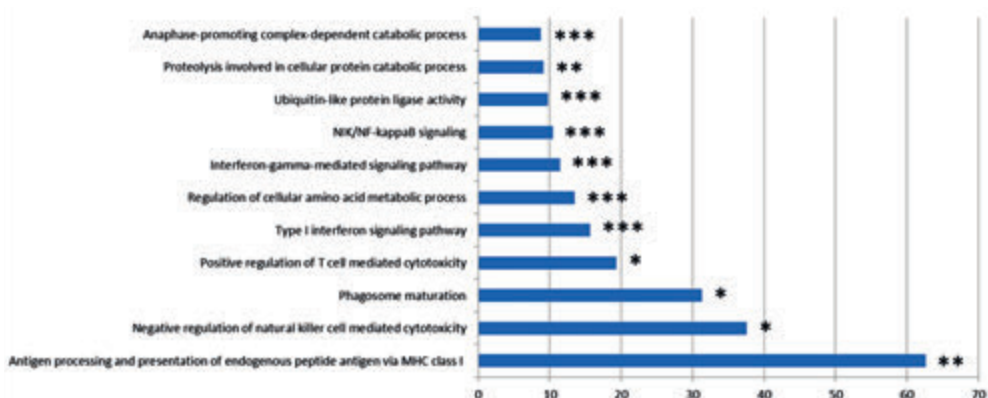


Figure 4: Biological processes involved in HPV-related vulvar squamous cell carcinoma (VSCC) DAVID GO enrichment analysis (biological process) of differentially expressed genes in HPV-related VSCC. The figure shows the most significant biological processes ($p < 0.01$) on the y-axis, and the fold enrichment on the x-axis. Asterisks indicate the p-values, * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$

3.3 HPV-independent VSCC precursors (dVIN)

3.3.1 Etiopathogenesis

HPV-independent VSCC has been associated with lichen sclerosus (LS), and occasionally with LSC, although the exact mechanism of this transformation remains unclear. The ‘scar cancer’ model has been proposed as a possible explanation, where chronic inflammation leads to repeated epithelial injury and incites malignant transformation [44].

Women with LS have a reported relative risk of 38.4 for the development of dVIN and VSCC [86]. Long-standing LS, and low compliance with high-dose potent topical steroid ointments have been associated with higher rates of (pre) malignant transformation [86].

The immune-microenvironment of LS resembles that of an autoimmune disorder, characterized by a high concentration of CD8⁺, and FOXP3⁺ T-cells, and T-cell receptor rearrangements; this probably provides a fertile ground for carcinogenesis [87-89].

TP53 mutations (missense, deletions, and nonsense) have been identified in both dVIN and VSCC, and have therefore been implicated in their pathogenesis [43, 90]. However, recent studies indicate that not all HPV-independent VSCC follow the *TP53* pathway, this will be discussed later.

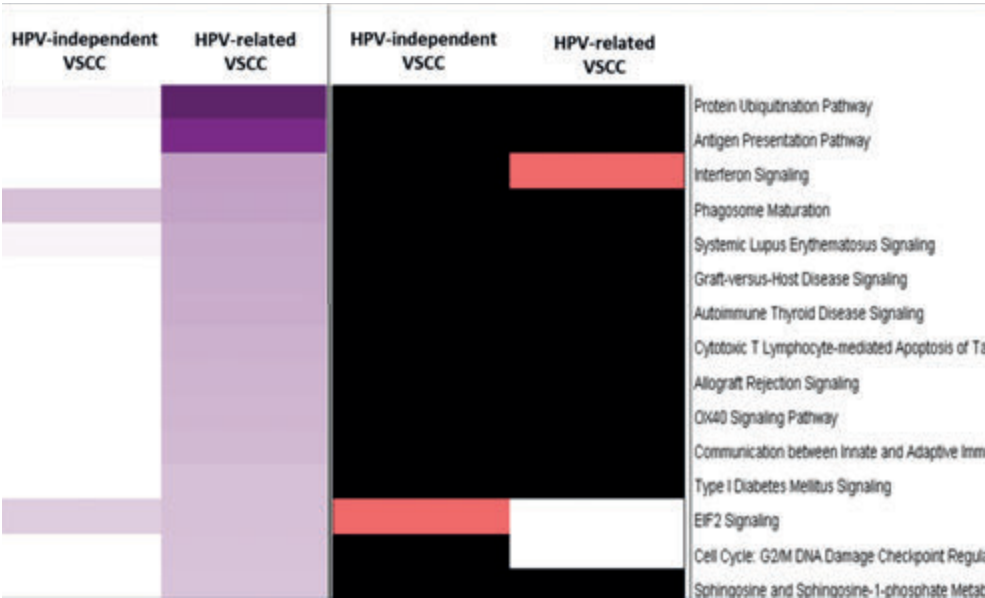


Figure 5: Canonical pathways involved in HPV-related vulvar squamous cell carcinoma (VSCC). Left: The top 15 canonical pathways regulated with statistical significance in HPV-related VSCC are shown, along with comparative regulation of these pathways in HPV-independent VSCC. Color-coding of the map corresponds to the $-\log(p\text{-value})$ of each canonical pathway, calculated by Fisher's exact test, right-tailed. Right: color-coding corresponds to the z-scores; red indicates predicted pathway activation, and black indicates that no predictions were available.

3.3.2 Clinical features

Although more common amongst post-menopausal women of 60 – 80 years, dVIN has also been reported in women < 40 years of age [48]. Clinically, dVIN often produces unifocal grey-white discolorations with a roughened surface, and less frequently whitish plaques, or nodules [48]. The clitoris is frequently involved. Perineal involvement is less common than in HSIL [91, 92]. Itching and burning sensations are common presentations, but occasionally, dVIN can be asymptomatic [43].

dVIN is less frequently diagnosed as a stand-alone lesion, than adjacent to VSCC. This has been attributed to under-diagnosis of dVIN, due to its subtle histological appearance, and rapid progression to VSCC [93]. The median interval between biopsy of dVIN and diagnosis of VSCC has been reported to be 43.5 months (range 8 – 102 months) [86]. VSCC arising in the background of dVIN has poorer prognosis (overall survival and disease specific survival), and recurs more commonly than VSCC associated with HSIL [91-93]. A history of prior, synchronous, or subsequent VSCC can be present in upto 85.7% of dVIN [86].

3.3.3 Histological features

Histological features of dVIN were extracted from 11 studies [27, 41, 43, 44, 48, 86, 94-98]. The 'differentiated' appearance of dVIN results from premature keratinization in the deeper epithelial layers, which is a consequence of disturbed maturation [Figure 6]. This overtly eosinophilic appearance due to premature keratinization can be readily identified even under low magnification [43, 44]. Unlike HSIL, nuclear atypia in dVIN is often limited to the basal layers [27, 41, 48].

The histological diagnosis of dVIN suffers from poor reproducibility [95]. A panel of experts from ISSVD consensually accepted basal layer atypia as the only essential diagnostic feature. Histological features arbitrated as 'strongly supportive' included basal layer hyperchromasia, basal layer mitoses, and large keratinocytes with abundant eosinophilic cytoplasm [94]. Features of disturbed maturation, such as individual cell keratinization, presence of squamous whorls and keratin pearls just above the basal layer, and cobblestoning have been reported to be particularly helpful for dVIN cases where nuclear atypia is difficult to discern [95, 96]. The histological features are listed in Table 1.

dVIN can sometimes be hard to distinguish from the reactive changes seen in LS, or LSC. Singh et al. therefore recommended that biopsies should include the interface between the lesion and normal skin, as this allows appreciation of the abrupt edges of dVIN, which lichenoid lesions lack [97].

Infrequently, dVIN exhibits full thickness moderate to severe atypia, similar to HSIL. These lesions are referred to as HSIL-like dVIN, or dVIN-with basaloid morphology, and can be distinguished from HSIL by the lack of koilocytes, and in certain cases, by the presence of focal conventional dVIN-like areas [98].

3.3.4 Immunohistochemical markers

Immunohistochemical markers studied in dVIN are presented below, and summarized in Table 2. This information was extracted from 20 studies [35, 48, 51, 53, 56, 58, 60, 61, 63, 64, 96-105]. The immunohistochemical markers have been categorized as per their sub-cellular location as nuclear, or cytoplasmic. The biological processes and canonical pathways associated with these markers are presented in Table S1 (supplementary document 2).

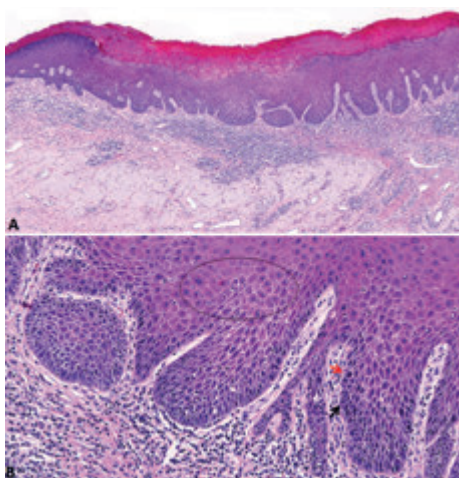


Figure 6: Differentiated vulvar intraepithelial neoplasia with characteristic histological features; hematoxylin-eosin (HE) stain. A. A widened epithelium with hyperkeratosis, parakeratosis, and an eosinophilic appearance can be appreciated under low magnification (original magnification 25X). B. Nuclear atypia, particularly prominent in the basal layers, can be appreciated under higher magnification (original magnification 200X). Angulated nuclei (black arrow) and mitotic figure (red arrow) are observed in the basal layer. Cobblestone appearance, individual cell keratinisation, and macronucleoli are present (circled area)

A. Nuclear markers

(i) p53: The underlying *TP53* mutations in dVIN can be reflected as over-expression/ mutation pattern, or null-pattern on p53-IHC [48, 99-100]. In mutation pattern, p53-staining in > 90% cells of the basal layer, along with suprabasal extension, ranging from lower one-third to full epithelial thickness is seen, and this is associated with missense mutations [Figure 7] [99, 100]. In null-pattern on the other hand, complete negativity is noted. This is seen in up to 27% cases of dVIN, and is associated with nonsense mutations [97].

A proportion of dVIN exhibits wild type (wt) p53-staining pattern [77]. Further delineation of the histological and molecular characteristics of these lesions is needed to determine whether they represent a subset of dVIN, or a distinct category of precursor of HPV-independent VSCC.

p53-IHC however, has limitations in distinguishing dVIN from lichenoid conditions. Increased p53-staining, named as 'p53-wt over-expression' can be seen in 5 – 61% cases of LS, and up to 40% cases of squamous hyperplasia, due to oxidative stress [99-103]. Moreover, p53-positivity has also been noted

in 'normal'-appearing vulvar skin, similar to the p53-signatures in the fallopian tube and endometrium, the significance of which is yet unknown [104].

(ii) p16: Complete negativity, to minimal focal staining with p16-IHC has been reported for dVIN [Figure 7] [51]. For dVIN histologically mimicking HSIL (HSIL-like dVIN), negative p16-IHC, and mutation-pattern or null-pattern expression with p53-IHC [Figure 7] helps distinguish it from HSIL [98].

(iii) Cyclin-D1: Nuclear cyclin-D1-expression has been reported to be mildly increased in dVIN, compared to normal vulvar epithelium. However, an almost identical staining pattern is seen in reactive vulvar disorders [53].

(iv) SOX2: Increased, strong SOX2-staining in the nuclei of basal and parabasal keratinocytes is seen in dVIN. In LS, only weak and occasional SOX2-staining from the basal to the upper epithelial layers is seen. SOX2-staining patterns in dVIN and LS have been reported to differ significantly ($p < 0.0001$) [56].

(v) GATA3: Partial to complete loss of nuclear expression of GATA3 in the basal layer, with or without loss in the parabasal layer has been reported for dVIN [58]. Since strong GATA3-staining has been noted in LS and LSC, GATA3 has been proposed as a potential diagnostic adjunct for dVIN [58].

(vi) hTERT: Nuclear staining with hTERT in dVIN has been reported to follow the distribution of the atypical cells in the epithelium, and to be significantly higher than that in LS [60].

(vii) Estrogen and progesterone receptors (ER α , ER β , and PR): In dVIN adjacent to VSCC in elderly patients, no nuclear expression of ER α was seen, whereas in LS, ER α -expression in the middle and lower-thirds of the epithelium was noted. For ER β , nuclear/cytoplasmic expression has been reported in both dVIN and LS. Scant, nuclear PR-expression has been reported in normal vulvar epithelium, LS, and dVIN [105].

(viii) Ki-67/MIB-1: Increased MIB-1-expression is seen in the basal and parabasal layers in dVIN. This can be helpful in distinguishing dVIN from LS, which usually shows only basal MIB-1-expression [35, 51].

(ix) p-S6: Increased nuclear p-S6-expression in the basal/parabasal layers, or extending across full epithelial thickness has been reported for dVIN. In contrast, p-S6-expression in LS was reported to be limited to the basal layers [61].

B. Cytoplasmic markers

(i) FSCN1: Diffuse cytoplasmic FSCN1-staining has been reported for dVIN, with some cases showing concurrent nuclear staining [63].

(ii) CK17: Intermediate to strong, diffuse, cytoplasmic CK17-expression, in the suprabasal layers to full epithelial thickness has been reported in dVIN [64]. CK17-expression has been reported to be significantly higher in dVIN compared to LS [96].

(iii) CK13: Weak cytoplasmic CK13-expression in the suprabasal or superficial epithelial layers, or complete loss of CK13-expression have been reported for dVIN [96]. For LS, patchy/diffuse CK13-expression of moderate intensity, in the suprabasal layers of the epithelium has been reported [96].

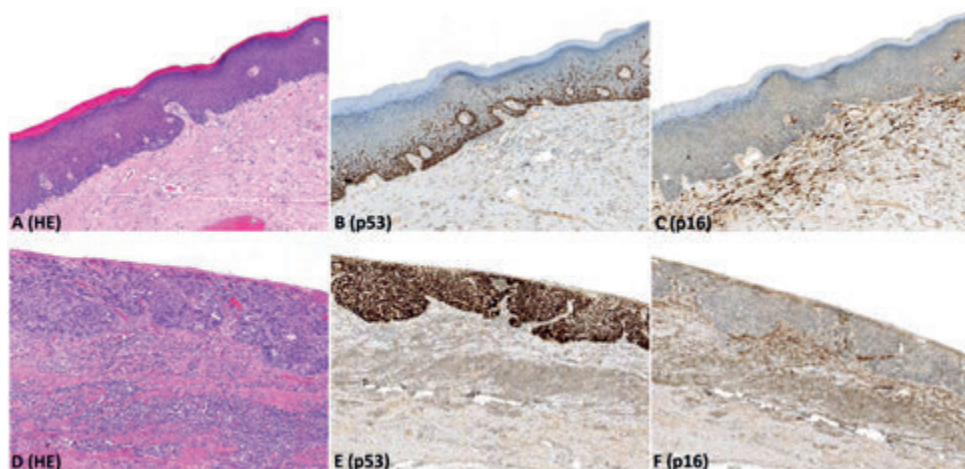


Figure 7: Differentiated vulvar intraepithelial neoplasia (dVIN), histology and p53 and p16 immunohistochemistry (IHC), original magnification 50X. A-C: dVIN with typical histology; A. Hematoxylin-Eosin (HE) stained section shows the characteristic histology of dVIN; B. On p53-IHC, mutation pattern staining, i.e. positive staining in the basal epithelial layers extending into the upper one-third of the epithelium is seen; C. Complete negativity is noted with p16-IHC; D-F: HSIL-like dVIN; D. HE stained section shows dVIN with full epithelial thickness atypia, which is more commonly noted in HSIL; E. Mutation pattern staining is noted with p53-IHC; F. Complete negativity is noted with p16-IHC

3.3.5 Molecular markers

A. Allelic imbalances, LOH, CNA

Loss of chr 2.4, 5.2, 3p, 13q, and 17p has been detected in both dVIN and HPV-independent VSCC [67, 68]. Loss of 3p and 17p has been most frequently reported [68-70, 73, 74, 102]. Alterations in chr 8p23.1, 8p23.3, and 8p11.22, and

chr 3 and chr 8 isochromosome formation (3p/8p loss with 3q/8q gain) have been more commonly detected in HPV-independent VSCC than in HPV-related VSCC [76]. Gains in chr 8q, and losses in chr 1p, 3p, and 8p have been reported to be more frequent in dVIN, than in HSIL [76].

B. Somatic mutations

Mutations of *TP53* are the most frequent somatic mutations in dVIN and HPV-independent VSCC [106, 107]. Frequency of *TP53* mutations in VSCC, in studies using next generation sequencing (NGS) or whole exome sequencing (WES), varied between 41 – 79% [77-80, 108]. Other somatic mutations that have been detected in VSCC, albeit in lower frequency, include *HRAS* (3 – 31%), *NOTCH1* (28 – 41%), *FGRF3* (4.8%), *CDKN2A* (11 – 36%), *FBXW7* (0 – 11%), *PIK3CA* (0 – 19%), *PPP2R1A* (3%), and *EGFR* [44, 77-80]. In lesions diagnosed histologically as dVIN, and showing wt-p53 expression on IHC, Nooij et al. detected mutations in *NOTCH1* (20% of cases) and *HRAS* (10% of cases) [77].

C. MSI

Bujko et al. could not detect MSI in HPV-independent VSCC, while Pinto et al. detected MSI in 27% of dVIN and 12% of LS cases in his study [109, 110].

D. Epigenetic changes

Hypermethylation of *CDKN2A*, stratifin, *RASSF1A*, *RASSF2A*, *TSP-1*, and *MGMT* have been reported for both dVIN and VSCC on ms-PCR, with a higher frequency in VSCC, than in dVIN [81, 82, 111].

3.3.6 GEO DataSet analysis

Three samples from the included dataset were identified as HPV-independent VSCC, in which the expression of 1158 probe sets (545 up and 613 down) significantly differed from the control. Of these, 382 probe sets (203 up and 179 down) were exclusive for HPV-independent VSCC. The associated biological processes, canonical pathways, and upstream regulators were identified using DAVID, and IPA, and are discussed below.

A. Biological Processes

The upregulated biological processes with the most significant p-values are depicted in Figure 8. These included biological processes associated with the

transcription factor CREB, metabolic processes, and reduced response to hormonal stimulation, and cellular senescence.

B. Pathways

Based on the $-\log(p\text{-values})$ of the differentially expressed genes, the associated canonical pathways were identified, and depicted in Figure 9. Metabolic pathways known to play a role in cell morphology, and embryonic development were most significantly upregulated. This is probably a reflection of the metabolic re-programming exhibited by solid tumors, to sustain proliferation and survival. Aryl hydrocarbon receptor signaling was the only signaling pathway in the top 5, which is known to down-regulate TGF- β mediated apoptosis.

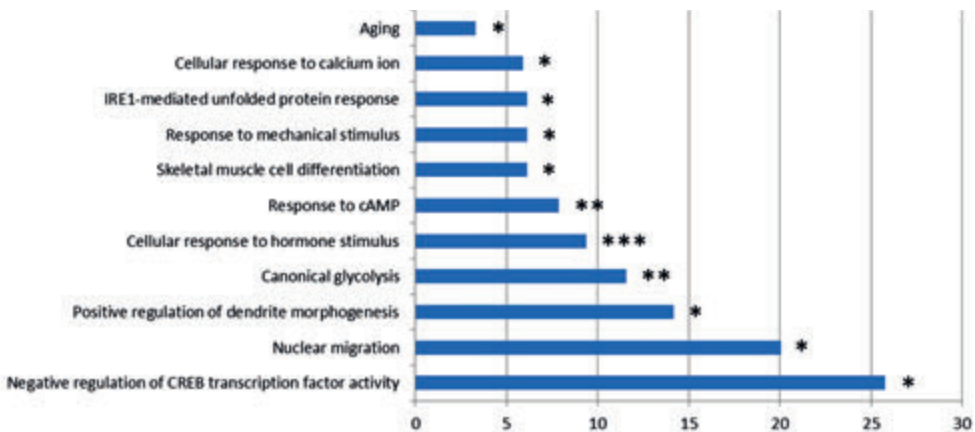


Figure 8: Biological processes involved in HPV-independent vulvar squamous cell carcinoma (VSCC). DAVID GO enrichment analysis (biological process) of differentially expressed genes in HPV-independent VSCC. The figure shows the most significant biological processes ($p\text{-value}<0.01$) on the y-axis, and fold enrichment on the x-axis. Asterisks indicate the p-values*, $p<0.01$; ** $p<0.001$; *** $p<0.0001$

C. Upstream regulators

Based on the z-scores, 57 upstream regulators (13 activated, 44 inhibited) were identified [Table S3, supplementary document 2]. Similarly to HPV-related VSCC, these comprised protein complexes, cytokines, enzymes, growth factors, G-protein coupled receptors, mature miRNAs, ligand-dependent nuclear receptors, transcription and translation regulators, and transporters. The top five activated upstream regulators included 4 transcription regulators i.e. MYCN proto-oncogene, NK2 homeobox 3 (NKX2-3), aryl hydrocarbon receptor nuclear

translocator (ARNT), and *SOX2*, and 1 mature microRNA (miR-125b-5p). MYCN, ARNT, and miR-125b-5p are known to regulate *TP53*, while *SOX2* is regulated by *TP53*.

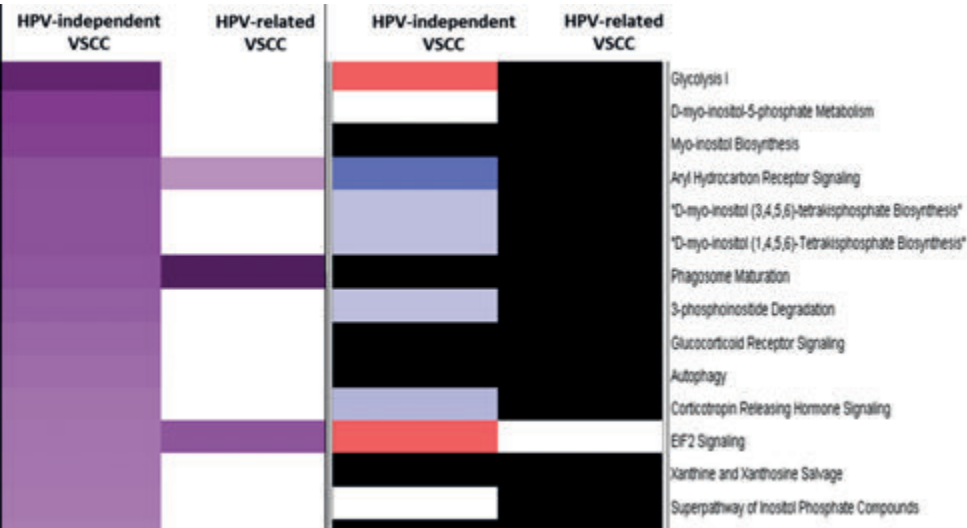


Figure 9: Canonical pathways involved in HPV-independent vulvar squamous cell carcinoma (VSCC). Left: The top 15 canonical pathways regulated with statistical significance in HPV-independent VSCC are shown, along with comparative regulation of these pathways in HPV-related VSCC. Color-coding of the map corresponds to the $-\log(p\text{-value})$ of each canonical pathway, calculated by Fisher's exact test, right-tailed. Right: Color-coding corresponds to the z-scores; red indicates predicted pathway activation, blue indicates predicted inhibition, and black indicates that no predictions were available

3.4 HPV-independent VSCC precursors (others)

Atypical verruciform lesions, with histological appearance distinct from dVIN, are other putative precursors of HPV-independent VSCC. Their etiopathogenesis, rates of malignant transformation, and molecular profiles remain largely unknown.

An example from this group is vulvar acanthosis with altered differentiation (VAAD) [112]. The typical histological features of VAAD are acanthosis with variable verruciform architecture, loss of granular layer with superficial epithelial pallor, and multilayered plaque-like parakeratosis [112].

Watkins et al. recently described a category of atypical verruciform lesions, bearing *PIK3CA* and *ARID2* mutations, instead of *TP53* mutations. These lesions,

with an exophytic, acanthotic, or verruciform architecture, and lacking the typical features of HSIL, or sufficient basal atypia for a diagnosis of dVIN, were named 'differentiated exophytic vulvar intraepithelial lesion (DEVIL)' [108].

4. Discussion

Concepts of VSCC precursors have significantly evolved since the first description in 1912, and a summary of current knowledge, with emphasis on histology and biomarkers, is presented in this review. As we included only English language literature, and articles with full-text availability, some relevant information may have been missed. The limited number of studies on immunohistochemical and molecular markers of VSCC precursors, and the small sample sizes in most of these, did not permit a meta-analysis. These are potential limitations of this review. Nevertheless, we discuss the important insights into VSCC and its precursors gained from the literature.

The WHO, ISSVD, and LAST committees have made commendable efforts to introduce and advocate standardized terminology for VSCC precursors. This allows better transfer of knowledge between clinicians, pathologists, and patients, and also ensures reproducibility in research. However, terminologies need modification in the face of newer information.

For dVIN, it is apparent that (i) malignant transformation is more frequent than HSIL, and (ii) occasionally it exhibits the histology typically associated with HSIL. In view of these 'high-grade' features, the legitimacy of the attribute 'differentiated' might be questioned. The merit of using HPV-independent/HPV-negative HSIL/high-grade VIN, in place of dVIN, may be deliberated.

Since dVIN is known to develop in patients with chronic vulvar dermatoses, biopsies should be performed for lesions recalcitrant to therapy, or with a suspicious clinical appearance, to rule out dVIN. Adequate sampling is also of utmost importance in these cases, as VSCC is often diagnosed adjacent to dVIN [27].

Regarding HPV-independent VSCC and dVIN, several facets of the pathogenesis still remain unknown. The association of LS and dVIN is widely reported in the literature, but a history of LS is not universally present in women with dVIN. Reports of p53-wt dVIN, and other putative precursors, e.g. DEVIL, indicate that

HPV-independent VSCC and its precursors constitute a heterogeneous category, with potentially different pathogenesis and natural history.

The clinical, histological, and molecular characteristics of the newly identified putative precursors need better delineation. Routine clinical photographs of vulvar lesions suspected to be premalignant may be useful in this context. One of the histological diagnostic criteria for DEVIL, i.e. 'insufficient basal atypia to warrant a diagnosis of dVIN', can be considered highly subjective. Furthermore, the malignant potential of these lesions needs to be established through prospective studies.

As an ancillary tool for the histological diagnosis of dVIN and HSIL, a reasonably wide assortment of immunohistochemical markers has been evaluated. Nevertheless, p53 and p16-IHC remain the most widely used in the clinical setting. Most studies on immunohistochemical markers included small numbers, and rarely did more than one study assess the same immunohistochemical marker. This has deterred their translation to the clinics.

Since p53 has limited value in discriminating dVIN from lichenoid lesions, immunohistochemical markers with higher specificity for dVIN, especially based on genetic and epigenetic profile, need to be explored. In view of the existence of cases of dVIN and HSIL with overlapping histology, routine use of p16-IHC to aid their accurate categorization should be considered, as the treatment and prognosis of both lesions differ significantly. Routine use of p16-IHC, as a surrogate marker of HPV-status, should also be considered for VSCC, as several studies have demonstrated poorer survival and higher recurrence rates for HPV-independent VSCC.

Studies on molecular characterization of VIN are limited, but they provide a few key findings. Intratumoral heterogeneity has been identified in HPV-related VSCC. For HPV-independent VSCC and precursors, genotypic subtypes (p53-wt category) with distinct mutational profiles have been identified. These data require validation in independent, larger cohorts, using whole genome sequencing. Epigenetic alterations, and the role of MSI are underexplored areas in VSCC, and may provide useful information for diagnosis, or prognostication. Our results from GEO analyses demonstrate the differences in pathways involved in HPV-related, and HPV-independent VSCC. Canonical pathways related to host-pathogen interactions were exclusively upregulated in HPV-related VSCC, whereas metabolic pathways influenced by *TP53* played a more

significant role in HPV-independent VSCC. The categories of upstream regulators also differed; pro-inflammatory cytokines were involved in HPV-related VSCC, whereas transcription regulators related to *TP53* were more operative in HPV-independent VSCC. A limitation of our results is the small sample size of the GEO dataset that was analyzed. Whether these processes and pathways are similarly involved in the precursors of the corresponding VSCC categories deserves exploration, as this may allow identification of potential therapeutic targets to improve clinical management.

5. Conclusion

The category of HPV-independent VSCC and its precursors needs better histological, immunohistochemical, and molecular delineation. Combination of advanced sequencing techniques, and leverage of bio-informatics can pave the path for molecular characterization of vulvar (pre)malignancies, and personalized treatment.

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4



CHAPTER 4

DIFFERENTIATED VULVAR INTRAEPITHELIAL NEOPLASIA (DVIN): THE MOST HELPFUL HISTOLOGICAL FEATURES AND THE UTILITY OF CYTOKERATINS 13 AND 17

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Abstract

Differentiated vulvar intraepithelial neoplasia (dVIN) is the precursor lesion of HPV negative vulvar squamous cell carcinoma (VSCC). The histopathological diagnosis of dVIN can be challenging, as it often resembles vulvar non-neoplastic epithelial disorders (NNED), especially lichen sclerosus (LS). We aimed to establish the most specific and reproducible histological features of dVIN, and assessed cytokeratin 13 (CK13) and cytokeratin 17 (CK17) immunohistochemistry as a diagnostic aid.

Consecutive cases of dVIN (n = 180) and LS (n = 105) from the period 2010 – 2013 were reviewed using a checklist of histological features. Each feature was recorded as 'present' or 'absent', and statistical comparison (dVIN vs LS) was made. Inter-observer agreement between two pairs of pathologists was assessed for a subset of cases of dVIN (n = 31), and LS and other NNED (n = 23). Immunohistochemistry with CK13, CK17, MIB1 and p53 was performed on dVIN, LS, and other NNED cases.

Macronucleoli, features of disturbed maturation, and angulated nuclei were significantly more common in dVIN than LS ($p < 0.001$). We found 'substantial agreement' for the diagnosis of dVIN (Kappa = 0.71). Macronucleoli and deep keratinisation had the highest agreement. In dVIN, the mean percentage of cells staining with CK13 was 15 and with CK17, this was 74. For LS, the mean percentage of cells staining with CK13 was 31, and with CK17, this was 41. By plotting receiver operating characteristic curves (ROC), an area under the curve (AUC) of 0.52 was obtained for CK13, and an AUC of 0.87 was obtained for CK17.

The most helpful histological features for diagnosing dVIN were macronucleoli, features of disturbed maturation, and angulated nuclei. Increased CK17 expression may have promise for supporting dVIN diagnosis.

1. Introduction

Vulvar intraepithelial neoplasia (VIN) is widely accepted as the precursor lesion of vulvar squamous cell carcinoma (VSCC) [1]. Vulvar squamous cell carcinoma (VSCC) arises via either a human papilloma virus (HPV)-associated pathway, or more commonly, via a mechanism independent of HPV, often being linked to chronic inflammatory conditions such as lichen sclerosus (LS) [1, 2]. Accordingly, two distinct subtypes of VIN are recognised: the HPV-associated high grade squamous intraepithelial lesion/usual VIN (HSIL/uVIN), and the non-HPV associated differentiated VIN (dVIN) [1]. High grade SIL is clinically identified by its multifocal, warty appearance and on histology by conspicuous cytological and architectural atypia [2]. Differentiated VIN, on the other hand, often produces ill-defined lesions, and on histology, notoriously mimics non-neoplastic epithelial disorders (NNED), particularly LS [1, 2]. As a result, dVIN is rarely (< 5% cases) identified in advance of a diagnosis of invasive malignancy, despite being the precursor lesion of the majority of VSCC [3]. Moreover, there is substantial inter-observer variability in the histological diagnosis of dVIN [4, 5]. In a recent study amongst vulva pathology experts, 'basal layer atypia' was the only criterion that met consensus to be 'essential' for dVIN diagnosis [6]. However, even this feature may not be readily appreciable in every case. The histological features of dVIN have been extensively described in the literature, but they have not been quantified so far [2, 5, 7].

In order to aid this difficult histological diagnosis, immunohistochemical markers p53 and MIB1 are commonly used, but both have limitations for making the distinction from NNED [2]. Increased p53 staining (overexpression) in the basal and parabasal layers is seen in dVIN, as a reflection of missense mutations of the *TP53* gene [2]. Additionally, 25 – 30% cases of dVIN show complete absence of p53 staining (null-pattern), due to nonsense mutations and deletions [8]. However, p53 overexpression also occurs in long standing LS and squamous hyperplasia, albeit as a consequence of ischemic stress [9-13]. The proliferation marker MIB1 can be increased in dVIN, as well as in NNED [14].

Recently, the diagnostic utility of the immunohistochemical markers cytokeratin 13 (CK13) and cytokeratin 17 (CK17) has been established for oral epithelial dysplasia [15-17]. Loss of CK13 along with expression of CK17 has been reported in (high grade) oral epithelial dysplasia [15-17]. Increased expression of CK17 has been reported for dVIN [14], but CK13 has not yet been explored for this lesion.

Through this study, we aimed to establish the histological features of dVIN, that are most helpful to reliably distinguish dVIN from LS. The immunohistochemical markers, CK13 and CK17, were evaluated as diagnostic adjuncts for dVIN. To the best of our knowledge, this is the first study to quantify the histological features of dVIN, and to assess both CK13 and CK17 for dVIN diagnosis.

2. Materials and Methods

Consecutive cases with a histological diagnosis of dVIN, LS, other NNED (e.g. lichen simplex chronicus, lichenoid inflammation, chronic non-specific inflammation, epithelial hyperplasia, hyperkeratosis) and VSCC, from the period 2010 – 2013 were identified from the electronic database of the Department of Pathology, Erasmus MC. All the data were anonymised. The slides of these cases were retrieved from the archives.

Our study comprised three steps: histological evaluation, reproducibility analysis, and assessment of immunohistochemistry. All the cases of dVIN and LS that were identified were included for the histological evaluation, and subsets of dVIN, LS, and NNED cases were used for the reproducibility and immunohistochemistry analyses. The details of each step are further elaborated below.

2.1 Histological evaluation

Histological evaluation was conducted on dVIN and LS, which is the closest and most difficult differential of dVIN. We formulated a checklist of histological features for dVIN, based on the literature [4, 5, 7]. The components of the checklist are listed and described below. For all cases of dVIN and LS, each of the features on the checklist was recorded as ‘present’ or ‘absent’. The statistical significance of each feature for the diagnosis of dVIN over LS was calculated.

Nuclear atypia: This included variation in nuclear size and shape, including angulated nuclei; abnormality of the nuclear chromatin, i.e., hyperchromatic or open chromatin; presence of macronucleoli, i.e. nucleoli visible at 100X magnification; and multi-nucleation.

Mitoses: The presence of suprabasal and/or atypical mitotic figures was noted. The number of mitotic figures per 5mm of the epithelium was counted.

Disturbed maturation: Disturbed maturation leads to premature keratinisation in the deeper layers of the epithelium, which was identified by a hypereosinophilic

appearance. Individual cell keratinisation, deep keratinisation, and deep squamous eddies (abortive pearls of keratin) were recorded as hallmarks of premature keratinisation. Cobblestone appearance of the epithelium, which is a combination of premature keratinisation with spongiosis, was recorded.

Architecture: Elongated (and anastomosing) rete ridges were noted.

Other features: Hyperkeratosis, parakeratosis, sub-epithelial hyalinisation and inflammatory cell infiltration were also recorded.

2.2 Reproducibility

A representative subset of 54 cases were selected by two pathologists, SDG and PEG. The set comprised 31 dVIN, 10 LS, and 13 other NNED cases. The cases were deliberately selected to provide a range of challenges. Thus, dVIN with classical histological appearances, as well as dVIN with ambiguous features, i.e. where the distinction between dVIN and LS was more difficult were included. Glass slides of these cases were independently assessed by two other pathologists, SK and VNH. They were asked to provide a diagnosis for each case, and adjudge the usefulness of the histological features for their diagnosis. The clinical history of the cases was not provided. No consensus training preceded the study.

The agreement between the pathologists for (i) the overall diagnosis and (ii) the presence of the individual histological features identified as most specific (from the checklist described above) was measured.

2.3 Immunohistochemistry

Immunohistochemistry was conducted on a subset of cases of dVIN, LS, and NNED. This set included cases found to have a good agreement for their diagnoses amongst pathologists in the reproducibility study, and additional ones.

Sections of 4µm thickness were prepared from formalin fixed paraffin embedded (FFPE) tissue. Cytokeratin 13 (clone KS-1A3, dilution 1:400, ThermoFisher), dual stain CK17-MIB1 (clone SP-95, Ready to use, Ventana) and p53 (BP53-11, Ready to use, Ventana) immunohistochemistry, with appropriate positive and negative controls were carried out according to manufacturer's instructions on Benchmark Ultra Immunostainer (Roche).

Defined areas adjudged to be dVIN were marked on the HE stained slides for accurate comparison with the slides stained with immunohistochemistry. Immunohistochemistry slides were scored by SDG, PEG, and SK on a multi-head microscope. The stains were analysed as described below.

CK13 and CK17: The percentage of cells showing cytoplasmic staining, the intensity of staining (weak, moderate, and strong) and their distribution in the epithelium was noted.

Receiver Operating Characteristic (ROC) curves were plotted for CK13 and CK17, using the percentage of cell stained, to assess their individual sensitivity and specificity for dVIN diagnosis. In order to assess whether these markers perform better when interpreted together, another ROC curve was derived.

MIB1: Increased MIB1 expression was noted as more than sporadic nuclear staining in the basal and/or suprabasal layers.

p53: Staining with p53 was recorded as overexpression, null pattern, or wild type. Intense nuclear staining in $\geq 50\%$ cells in the lower 1/3rd of the epithelium, occasionally extending to the suprabasal layers was considered overexpression. Nuclear staining of weak to moderate intensity, in $< 50\%$ cells in the lower 1/3rd of epithelium was noted as wild type. Complete absence of staining was noted as null pattern.

2.4 Statistical analysis

Data analysis was done with IBM SPSS Statistics 24 (SPSS, Chicago, IL, USA). Independent sample's t-test was used for parametric data and Chi-square (χ^2) test for non-parametric data to deduce the p value. A p value < 0.05 was considered statistically significant. Inter-observer agreement was measured with Cohen's Kappa. Kappa (κ) was interpreted as < 0.20 = poor, $0.21 - 0.40$ = fair, $0.41 - 0.60$ = moderate, $0.61 - 0.80$ = substantial, and $0.81 - 1.00$ = almost perfect agreement.

3. Results

From the archives, 180 cases of dVIN, 105 cases of LS, and 126 cases of NNED were identified. Of the 180 cases of dVIN, 61 were isolated dVIN, and 119 were identified next to VSCC. Fifty nine percent (36/61) of the isolated dVIN had a history of VSCC.

For histological evaluation, 180 cases of dVIN, and 105 cases of LS were included.

3.1 Histological evaluation

Nuclear atypia: All cases of dVIN showed nuclear atypia, albeit in varying extents. Some variation in nuclear size and shape could be noticed under low magnification (100X) in 63% (114/180) of dVIN. Abnormalities of nuclear chromatin (hyperchromatic or open chromatin) were present in all dVIN cases. Angulated nuclei, seen in 66% (119/180) of dVIN, and macronucleoli, seen in 65% (118/180) of dVIN had the strongest statistical significance ($p < 0.001$).

Mitoses: Atypical mitoses and suprabasal mitoses were noted more frequently than a mitotic count $> 5/5\text{mm}$ in dVIN.

Disturbed maturation: Individual cell keratinisation was present in 92% (165/180), deep keratinisation in 78% (141/180), and deep squamous eddies in 61% (110/180) of dVIN cases. Cobblestone appearance of the epithelium was noted in 83% (149/180) of dVIN cases. All of these features had strong statistical significance for dVIN ($p < 0.001$).

Architecture: Elongated rete ridges were present in 63% (114/180) of dVIN ($p < 0.01$) and appeared to be anastomosing in 20% (37/180) of cases.

Other features: Parakeratosis was noted in 73% (132/180) of dVIN ($p < 0.01$).

An overview of the histological evaluation is given in Table 1, and the histological features are illustrated in Figures 1 and 2.

3.2 Reproducibility

We found 'substantial' inter-observer agreement [$\kappa = 0.71$, Standard error (SE) = 0.11, 95% Confidence interval (CI): 0.51 - 0.95] on the selected subset of cases for the diagnosis of dVIN. Out of all the histological features evaluated from the checklist, 'substantial' agreement were obtained for macronucleoli ($\kappa = 0.75$), deep keratinisation ($\kappa = 0.71$), deep squamous eddies ($\kappa = 0.68$), individual cell keratinisation ($\kappa = 0.66$), mitotic count $> 5/5\text{mm}$ ($\kappa = 0.64$), and angulated nuclei ($\kappa = 0.60$). The histological features with 'substantial' and 'moderate' agreement are listed in Table 1 of supplementary material.

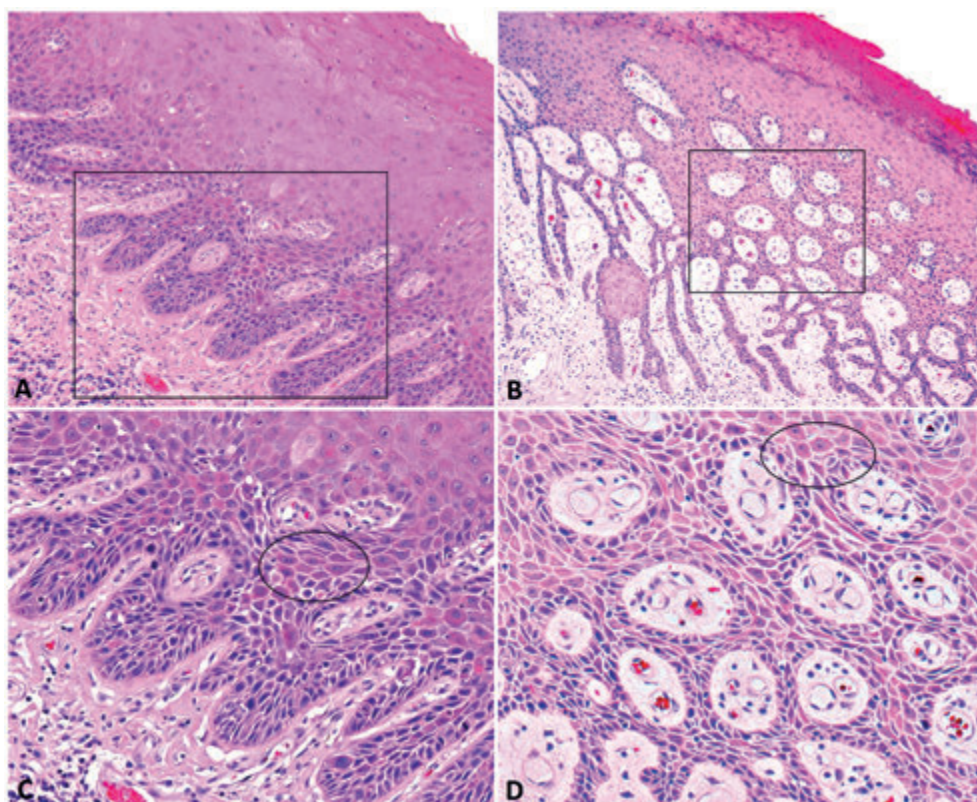


Figure 1: Example of differentiated VIN with characteristic features (HE stain), low magnification appearance in A and B, with corresponding higher magnification images in C and D. A. A widened epithelium with parakeratosis, and elongated rete ridges is seen. Nuclear atypia, premature keratinisation, and cobblestone appearance are apparent (original magnification 50X); B. Elongated rete ridges, a deep squamous eddy, nuclear atypia, and parakeratosis can be identified under low magnification (original magnification 50X); C. Under higher magnification, macronucleoli can be seen. Angulated nuclei, individual cell keratinisation and cobblestone appearance (circled area) can be better appreciated (original magnification 100X); D. Atypical cells with both open chromatin and hyperchromatic patterns are seen. There is cobblestone appearance (circled area) and individual cell keratinisation (original magnification 100X).

3.3 Immunohistochemistry

Immunohistochemistry with p53, CK13 and dual stain CK17-MIB1 was conducted on an initial set of 24 cases of dVIN, 9 cases of LS, and 8 cases of NNED. For further evaluation of CK13 and CK17, these stains were carried out on an additional set comprising 30 cases of dVIN, 5 cases of LS, and 22 cases of NNED.

Cytokeratin 13

For LS and other NNED cases, the percentage of cells staining with CK13 was higher compared to dVIN, and the staining intensity was stronger [Figure 3].

Differentiated VIN (n = 54): In 15% (8/54) of dVIN, there was a complete lack of CK13 staining. Patchy, weak staining in the suprabasal layers was seen in 67% (36/54); and diffuse, weak staining in the superficial layers was seen in 18% (10/54). The mean percentage of cells positive for CK13 was 15 [95% CI: 9.4 - 20.5].

Lichen sclerosus (n = 14): None of the LS cases showed complete lack of CK13 staining. In 79% (11/14) diffuse staining of moderate intensity was noted in the suprabasal layers; and in 21% (3/14), patchy staining of moderate intensity was noted in the suprabasal layers. The mean percentage of cells positive for CK13 was 31 [95% CI: 11.4 - 50].

NNED (n = 30): Complete lack of CK13 staining was also not seen in any of the NNED cases. In 57% (17/30) of cases, diffuse staining of moderate intensity was noted in the suprabasal layers; in 43% (13/30), patchy staining of moderate intensity was noted in the suprabasal layers. The mean percentage of cells positive for CK13 was 39 [95% CI: 23.6 - 53.8].

Table 1: Evaluation of histological features (dVIN vs LS)

Histological features	dVIN (n = 180)	LS (n = 105)	p-value
	Number (%)	Number (%)	
A. Nuclear atypia			
Obvious under low power (100x)	114 (63)	0 (0)	0.01
Angulated nuclei	119 (66)	8 (8)	0.001
Chromatin pattern			
Open	96 (53)	6 (6)	0.01
Hyperchromatic	84 (47)	10 (10)	0.01
Macronucleoli	118 (65)	24 (23)	0.001
Multinucleation	129 (72)	65 (62)	0.20
B. Mitotic figures			
Mitotic count >5/5mm	67 (32)	20 (19)	0.003
Atypical mitoses	102 (57)	0 (0)	< 0.001
Suprabasal mitoses	122 (68)	13 (12)	< 0.01

Histological features	dVIN (n = 180)	LS (n = 105)	p-value
	Number (%)	Number (%)	
C. Disturbed maturation			
Individual cell keratinisation	165 (92)	21 (20)	< 0.001
Deep keratinisation	141 (78)	4 (4)	< 0.001
Deep eddies	110 (61)	0 (0)	< 0.001
Cobblestone appearance	149 (83)	12 (11)	0.001
D. Architecture			
Elongated Rete Ridges	114 (63)	7 (7)	0.01
Elongated and anastomosing	37 (21)	0 (0)	0.01
E. Others			
Inflammatory response			
Scanty/focal	41 (23)	17 (16)	0.25
Moderate	82 (46)	51 (49)	
Marked	57 (32)	37 (35)	
Sub-epithelial Hyalinisation	124 (69)	81(77)	0.10
Hyperkeratosis	125 (69)	77 (73)	0.58
Parakeratosis	132 (73)	56 (53)	0.009

Cytokeratin 17

For dVIN, the percentage of cells staining with CK17 was higher compared to LS and other NNED cases, and staining intensity was stronger [Figure 3].

Differentiated VIN (n = 54): Diffuse, strong staining, across the full thickness of the epithelium was seen in 33% (18/54). The rest of the dVIN showed diffuse, moderate to strong staining in the suprabasal layers in 56% (30/54) of cases, and patchy, strong staining in the superficial layers in 11% (6/54) of cases. The mean percentage of cells positive for CK17 was 74 [95% CI: 68.5 - 81.2].

Lichen sclerosus (n = 14): None of the cases of LS showed diffuse, strong staining across full epithelial thickness. Patchy, weak staining in the suprabasal layers was seen in 64% (9/14), and diffuse, weak staining in the superficial layers in 36% (5/14). The mean percentage of cells positive for CK17 was 41 [95% CI: 18.4 - 51.6].

NNED (n = 30): Complete absence of CK17 staining was noted in 57% (17/30) of NNED cases. Patchy, weak staining in the suprabasal layers was seen in 27% (8/30), and diffuse, weak staining in the superficial layers was seen in 16% (5/30). The mean percentage of cells positive for CK17 was 19 [95% CI: 7.8 - 31.1].

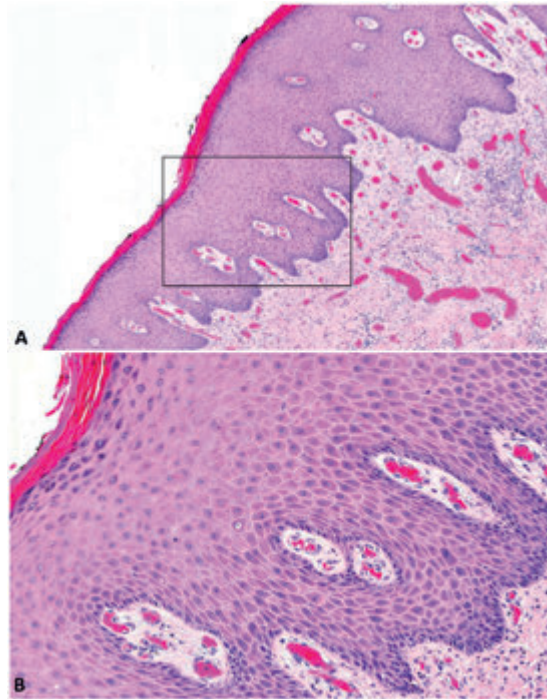


Figure 2: Example of differentiated VIN with relatively subtle histological features (HE stain) A. Nuclear atypia cannot be easily discerned under low magnification (in contrast to Figures 1A and 1C). A widened epithelium, parakeratosis, some elongation of rete ridges, and mildly increased cellularity are seen (original magnification 50X); B. Under higher magnification, nuclear atypia with open chromatin pattern, macronucleoli, individual cell keratinisation, and cobblestone appearance are detectable (original magnification 100X).

On computing ROC curves, CK13 had an area under the curve (AUC) of 0.52 [SE 0.06, 95% CI: 0.40 – 0.64], while CK17 had an AUC of 0.87 [SE 0.04, 95% CI: 0.80 – 0.94]. The combination of both stains, i.e., when CK13 and CK17 immunohistochemistry was interpreted together, showed an AUC of 0.76, [SE 0.05, 95% CI: 0.70 – 0.87] [Figure 4].

MIB1

Increased MIB1 was noted in 67% (36/54) dVIN, 36% (5/14) LS, and 47% (14/30) other NNED cases; the details are elaborated in Table 2 of supplementary materials.

p53

Differentiated VIN (n = 24): Overexpression of p53 was seen in 45% (11/24), null pattern in 13% (3/24), and wild-type expression in 42% (10/24). Cases with null pattern and overexpression of p53 were considered positive for dVIN [Figure 3]

Lichen sclerosis (n = 9): None of the cases of LS showed the 'null pattern'. Overexpression of p53 was seen in 33% (3/9) and wild-type expression in 67% (6/9) of LS cases [Figure 3].

NNED (n = 8): All the NNED cases had wild type p53 expression.

The details of p53 expression are elaborated in Table 3 of supplementary materials.

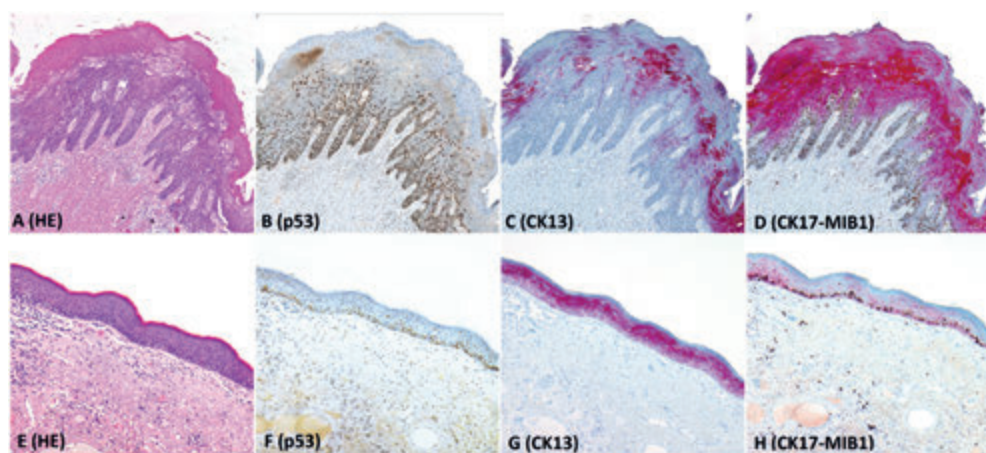


Figure 3: Immunohistochemistry in differentiated VIN (A-D) and Lichen sclerosis (E-H). A. Differentiated VIN, HE stain; B. Overexpression of p53; C. Weak, patchy CK13 staining; D. Strong and diffuse CK17 expression, with increased MIB-1; E. Lichen sclerosis, HE stain; F. Wild-type p53 expression; G. Diffuse staining of moderate intensity with CK13; H. Very weak, patchy CK17 staining with increased MIB-1.

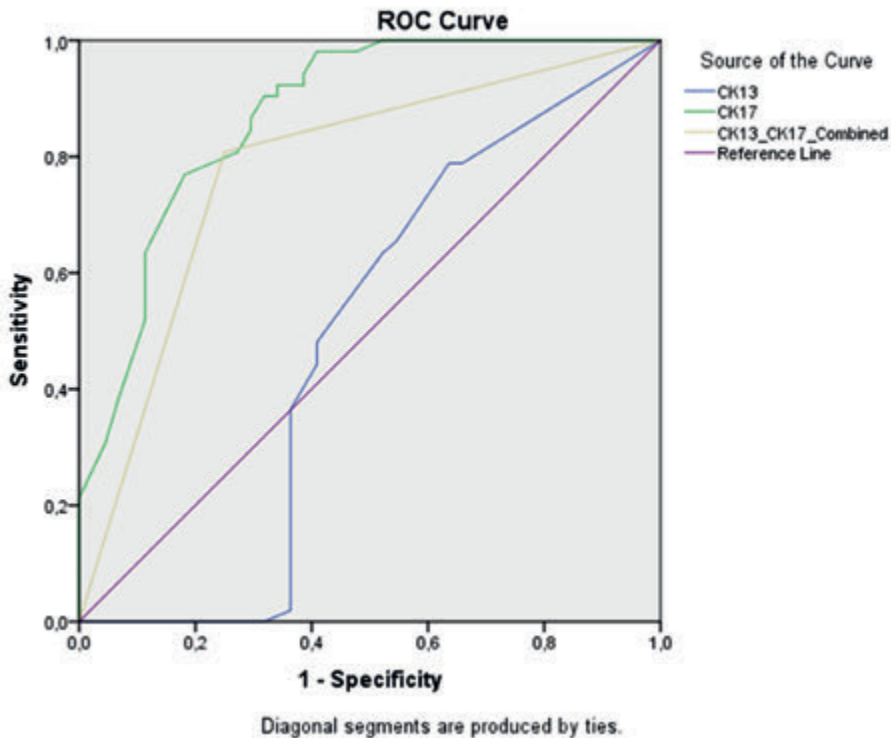


Figure 4: ROC curves for CK13 and CK17 immunohistochemistry for the diagnosis of dVIN. The green line represents CK17 and the blue line represents CK13, when they are interpreted individually. The yellow line represents the ROC curve when CK13 and CK17 are interpreted together. Area under the curve (AUC) for CK13: 0.52, CK17: 0.87, CK13 and CK17 combined: 0.76.

4. Discussion

Differentiated VIN was first recognised as a precursor lesion of VSCC in 1961 [2]. Over the years, a plethora of descriptive terminology (e.g. vulvar dystrophy/hyperplasia with atypia) led to incorrect categorisation of this lesion. Recent studies report that the non-HPV related pathway contributes to around 80% of all VSCC [1, 2]. However, dVIN comprises only 2 – 29% of standalone VIN diagnoses [2, 18-20]. This implies that dVIN maybe under-recognised, underlining the need for well-defined diagnostic criteria.

Clinically, dVIN is known to present with vague grey-white discoloration [2-5], on the background of long-standing LS, and shows subtle histological features which are often difficult to distinguish from LS. However, dVIN is known to

progress rapidly to invasive carcinoma, with a reported median interval of 28 months [5, 18, 21]. Thus, dVIN is often only recognised on histology adjacent to VSCC, or on follow up biopsies. Literature describes the presence of dVIN next to VSCC in up to 40% of cases [21]. In our study, dVIN was identified next to 49% of VSCC cases.

To facilitate the reliable diagnosis of dVIN, we set out to quantify its individual histological features. We found that nuclear atypia, the sine qua non for dVIN diagnosis, could be discerned under low power in only 63% of cases. Among the components of nuclear atypia, macronucleoli and angulated nuclei were the most specific. Both these features had 'substantial' inter-observer agreement in terms of relevance for a dVIN diagnosis. They can therefore be useful to discriminate the nuclear atypia of dVIN from the reactive nuclear enlargement seen in LS and other NNED. Abnormality of the nuclear chromatin was noted in all dVIN cases, with hyperchromatic or an open chromatin pattern occurring with almost equal frequency. A mitotic count of $> 5/5\text{mm}$ and atypical mitoses, although specific for dVIN, were seen less frequently. Multinucleation, a common feature of dVIN, was also seen regularly in LS and other NNED cases, and thus lacked statistical significance for dVIN diagnosis.

Disturbed maturation in the form of premature keratinisation in the basal or parabasal layers is a morphological reflection of the underlying pathology. Manifestations of disturbed maturation (individual cell keratinisation, deep keratinisation and deep eddies) were commonly noted in dVIN. The features of disturbed maturation had the second highest level of agreement amongst our pathologists, next in importance only to macronucleoli for dVIN diagnosis. Cobblestone appearance of the epithelium [22], elongated (\pm anastomosing) rete ridges and parakeratosis could also be reproducibly identified, and these features should be regarded as important pointers towards the diagnosis of dVIN, especially in cases where nuclear atypia cannot be easily discerned. Spongiotic changes of the epithelium seen in NNED should not be mistaken for the cobblestone appearance, as the latter is always accompanied by evidence of disturbed maturation. We found individual cell keratinisation, deep keratinisation, cobblestone appearance, and parakeratosis to occur more frequently than suprabasal mitosis or abnormal mitotic figures in dVIN. With this study, we hope to highlight the importance of detailed scrutiny of these supporting features.

We noticed that dVIN can show a whole spectrum of morphological features. However, even in the most subtle cases with minimal nuclear atypia, alteration of cellularity and alignment of nuclei with individual cell keratinisation and parakeratosis are present. A link between the particulars of the histological appearance and progression to VSCC, could potentially be explored. This detailed description and quantification of the morphological features is primarily intended to guide the general pathologist to recognise dVIN, particularly in the dubious cases where the difference from LS may not be apparent.

Immunohistochemistry with p53 and MIB1 is often used to support the diagnosis of dVIN. However, no universal cut-off exist for the interpretation of these stains, and thus, the distinction between wild type p53 expression and p53 overexpression may not be easy to make. We found the p53 null pattern to be specific for dVIN, but only a minority of dVIN show this pattern. A proportion of both dVIN and LS showed wild-type expression, and utility of p53 can be limited in these cases.

The second step of this study was to evaluate CK13 and CK17 as potential diagnostic adjuncts for dVIN. Cytokeratins are cell type specific intermediate filament proteins and their expression is altered in abnormalities of cellular differentiation. Expression patterns of cytokeratins 8, 10, 13 and 14 in VSCC were studied in 1995 by Ansink et al [23]. They noted CK13 expression in well differentiated VSCC, as well as in the normal epithelium of labium minus. Recently, there has been a lot of interest in cytokeratin research, particularly for (high grade) oral dysplasia.

Cytokeratin 13 is expressed physiologically from the prickle cell layer (third basal layer) to the keratinised layer (surface) in normal oral mucosa [24, 25]. Progressive loss of CK13 with increasing grades of dysplasia has been demonstrated in the oral cavity, cervix and oesophagus [25-32]. Cytokeratin 17 is a basal/myoepithelial cell keratin which is not expressed under physiological conditions in oral mucosa or perianal skin [16, 33, 34]. Increased CK17 expression has been reported in oral, cervical, and anal intraepithelial neoplasia [17, 31, 33, 34]. There is little information about CK17 expression in normal vulvar tissue. In dVIN, increased CK17 expression has been reported on in a single study [14].

We found CK13 expression to be lower in dVIN compared to LS and other NNED. On the other hand, CK17 expression was higher in dVIN than in LS and other NNED. From analyzing the ROC curves, increased CK17 expression showed

better sensitivity and specificity than CK13 loss for dVIN. Similar to the findings of Podoll et al [14], diffuse CK17 staining across full epithelial thickness or in the suprabasal layers was found to be strongly supportive of a dVIN diagnosis. Complete lack of CK13 staining was specific for dVIN, but this occurred in only 15% of cases. In some cases, CK17 immunohistochemistry may be equivocal, for example, patchy, strong staining, or diffuse staining in the superficial layers only. In this situation, a reduced expression or a complete lack of CK13 staining can offer additional support for the diagnosis of dVIN.

Our study, in common with most retrospective studies, has some limitations; a selection bias cannot be ruled out. For testing the reproducibility a limited number of cases selected by two pathologists was included, and the two other participants were experienced pathologists from the same institute. Thus, our results may not entirely reflect daily diagnostic practice. External validation studies, with more cases and participants from other centres will follow. With respect to immunohistochemistry; more extensive research on the expression of CK17 in vulvar skin and mucosa is necessary to establish its relevance in practice

Despite the limitations, we have attempted to describe here the most helpful histological features to enable the diagnosis of dVIN. Increased CK17 expression has potential as a diagnostic adjunct for dVIN, and deserves further exploration in this context.

5. Conclusion

Macronucleoli and angulated nuclei should alert the pathologist to consider the diagnosis of dVIN. Disturbed maturation and cobblestone appearance are other specific and reproducible features of dVIN, and may be of particular use where nuclear atypia is less prominent. Increased CK17 expression may have promise as an adjunct to histology for discriminating dVIN from close differentials.

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5



CHAPTER 5

HISTOLOGICAL DIAGNOSIS OF DIFFERENTIATED VULVAR INTRAEPITHELIAL NEOPLASIA (DVIN) REMAINS CHALLENGING – OBSERVATIONS FROM A BI-NATIONAL RING-STUDY

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Abstract

Differentiated vulvar intraepithelial neoplasia (dVIN) is a premalignant lesion that is known to progress rapidly to invasive carcinoma. Accurate histological diagnosis is therefore crucial to allow appropriate treatment. To identify reliable diagnostic features, we evaluated the inter-observer agreement in the histological assessment of dVIN, among a bi-national, multi-institutional group of pathologists.

Two investigators from Erasmus MC selected 36 hematoxylin-eosin stained glass-slides of dVIN and no-dysplasia, and prepared a list of 15 histological features of dVIN. Nine participating pathologists (i) diagnosed each slide as dVIN or no-dysplasia, (ii) indicated which features they used for the diagnosis, and (iii) rated these features in terms of their diagnostic usefulness. Diagnoses rendered by > 50% participants were taken as the consensus (gold-standard). p53-immunohistochemistry (IHC) was performed for all cases, and the expression patterns were correlated with the consensus diagnoses. Kappa (κ)-statistics were computed to measure inter-observer agreements, and concordance of the p53-IHC patterns with the consensus diagnoses.

For the diagnosis of dVIN, overall agreement was moderate ($\kappa = 0.42$), and pairwise agreements ranged from slight ($\kappa = 0.10$) to substantial ($\kappa = 0.73$). Based on the levels of agreement and ratings of usefulness, the most helpful diagnostic features were parakeratosis, cobblestone appearance, chromatin abnormality, angulated nuclei, atypia discernable under 100X, and altered cellular alignment. p53-IHC patterns showed substantial concordance ($\kappa=0.67$) with the consensus diagnoses.

Histological interpretation of dVIN remains challenging with suboptimal inter-observer agreement. We identified the histological features that may facilitate the diagnosis of dVIN. For cases with a histological suspicion of dVIN, consensus-based pathological evaluation may improve the reliability of the diagnosis.

1. Introduction

Differentiated vulvar intraepithelial neoplasia (dVIN) is the immediate precursor of human papillomavirus (HPV)-independent vulvar squamous cell carcinoma (VSCC), and is postulated to develop on the background of chronic dermatoses, driven by *TP53* mutations [1-4]. Recent literature suggests that dVIN has an accelerated rate of progression to VSCC (median interval: 41.4 months), and a high recurrence rate [5-7]. In view of this, current treatment guidelines [8, 9] recommend surgical excision of lesions that are histologically diagnosed as dVIN. Evidently, accurate histological diagnosis is crucial to allow appropriate patient management.

On histology, distinguishing dVIN from dermatoses, such as lichen sclerosus (LS), can present a challenge, as dVIN often exhibits subtle atypical features that mimic the reactive changes seen in chronic dermatoses [10-12]. The difficulty of diagnosing dVIN can give rise to diagnostic variability, which has the potential to critically affect treatment decisions [13, 14].

Although the diagnostic difficulty of dVIN has been acknowledged in literature [2-5], there is insufficient data on the inter-observer agreement in the histological assessment. In a previous study, we established the features that helped to reliably distinguish dVIN from LS, and could be interpreted with substantial agreement by pathologists at our center [15]. However, it remains to be determined whether similar level of agreement can be achieved between pathologists from different practice settings.

In the current study, therefore, we evaluated the inter-observer agreement for the diagnosis, and in the interpretation of histological features of dVIN, among a bi-national, multi-institutional group of pathologists. We also assessed the perception of the pathologists regarding the diagnostic usefulness of the histological features. Our aim was to thereby identify reliable diagnostic features that may facilitate the diagnosis of dVIN. In addition, we correlated the immunohistochemical expression patterns of p53 with the consensus histological diagnoses, as this marker is frequently used as an ancillary tool to support the histological diagnosis of dVIN.

2. Materials and methods

2.1 Study design

For the purpose of this study, two investigators (SDG and PCEG) identified all vulvar lesions from 2010 – 2013, from the electronic records of the Department of Pathology, Erasmus MC. All of these lesions were from patients who underwent vulvar biopsies or excisions at Erasmus MC. Hematoxylin-eosin (HE) stained slides of these lesions were retrieved from the archives, and the histology was reviewed by these investigators.

From this series, the investigators selected a set of 36 slides for inclusion in this study. The selection was enriched for lesions regarded as dVIN by the investigators on histology review, since the aim was to evaluate inter-observer agreement in dVIN. Furthermore, to provide a range of challenges to the participants, the selection was prepared in a way to include – (i) lesions adjacent to VSCC, as well as, standalone lesions, and, (ii) lesions with classical histology, which were diagnostically straightforward, as well as, lesions where the distinction between dVIN and no-dysplasia could be difficult. The selection did not comprise any slides with invasive carcinoma, as presence of VSCC in the adjacent epithelium can be considered by pathologists as a diagnostic clue for dVIN [14].

Therefore, of the 36 selected slides, 25 contained lesions adjacent to VSCCs, and 11 contained standalone lesions. The investigators had judged 26 (72%) slides as dVIN and 10 (28%) slides as no-dysplasia, comprising 6 lichen sclerosus and 4 non-specific reactive lesions. The investigators perceived 67% of the diagnoses as straight-forward and 33% as difficult.

The original diagnoses of these slides, or the diagnoses rendered by the investigators on review were not used for the analyses. For each slide, the diagnosis rendered by > 50% of the participants was taken as the consensus diagnosis / gold-standard.

For de-identification, all slides were re-labeled with opaque stickers bearing a random number. No serial sections were prepared. To ensure that all pathologists evaluated identical areas, the regions of interest were marked on the glass slides with red lines.

For all included slides, immunohistochemistry (IHC) was conducted with (i) p16 (E6H4-clone, Ventana), to confirm that the selection did not contain any

HPV-related lesion, and with (ii) p53 (Ventana), to correlate with the consensus diagnosis. The IHC-protocol is detailed in supplementary document 1. IHC slides were read only by the investigators and were not provided to the participants. IHC was scored and interpreted as described below:

p16-IHC patterns were scored as block-type or non-block type (patchy), following the guidelines of The Lower Anogenital Squamous Terminology Standardization Project (LAST) [16]. Block-type p16-expression is considered to be indicative of a high-risk HPV-infection [16]. This pattern was not present in any slide, confirming that the selection did not contain any HPV-related lesion.

p53-IHC patterns were scored as p53-mutant or p53-wild-type, following recent literature [17, 18]. p53-mutant patterns include basal to parabasal / diffuse overexpression, basal overexpression, null-pattern, or cytoplasmic expression, and these have been reported to strongly correlate with the presence of *TP53* mutations [17-19]. Presence of any of these patterns, therefore, can be considered supportive of a histological diagnosis of dVIN.

p53-wild-type pattern, i.e. scattered, heterogeneous, basal / parabasal expression, is primarily seen in non-dysplastic lesions. However, this pattern has been also occasionally observed in dVIN [15, 20-23]. Hence, a p53-wild type pattern does not preclude a histological diagnosis of dVIN. p53 patterns observed in our slides are presented in Results.

Next, a list of histological features of dVIN was compiled from previously published literature [13-15, 24-26], and incorporated into an assessment form. These comprised –

Features of nuclear atypia: (i) atypia discernable under 100X magnification; (ii) angulated nuclei; (iii) macronucleoli, i.e. nucleoli visible under 100X magnification; (iv) chromatin abnormality (open or hyperchromatic pattern); (v) multinucleation; (vi) suprabasal mitoses; (vii) atypical mitoses; and (viii) mitotic count > 5/5mm;

Features of disturbed maturation / architecture: (i) individual cell keratinization; (ii) deep keratinization; (iii) deep squamous eddies, i.e. abortive pearls of keratin; (iv) cobblestone appearance, i.e. combination of premature keratinization and spongiosis; (v) elongated and / or anastomosing rete ridges; (vi) altered cellular alignment; and (vii) parakeratosis.

2.2 Participants

Pathologists who attend the gynecological-pathology working group of the Rotterdam-region were invited to participate. HE-stained glass slides were circulated among the participants for histological assessment. Instructions and forms for the assessment (supplementary document 2) were sent to the participants electronically. Clinical information, original diagnoses, or IHC results were not provided. There was no consensus meeting prior to the assessment to determine any diagnostic criteria. To allow the participants to interpret the histological features in light of their own experience, detailed instructions regarding this was not provided. For measuring 5mm to assess the mitotic count, participants could use an eye-piece graticule, or the field-diameter of the eye-pieces of their microscopes. Since the measure of 5mm was an arbitrarily chosen cut-off, a rough estimate of this measurement was considered sufficient. The participants were masked from each other's assessments. Information regarding the nature of practice (academic / non-academic), country of practice, and length of practicing experience was gathered from the participants.

2.3 Histological assessment

Participants were asked to independently examine the areas marked on the slides, and:

- i. provide a diagnosis as – dVIN or no-dysplasia;
- ii. score the histological features (listed above) as – not present or present, and if present, indicate whether they were useful, or very useful for the diagnosis of dVIN; and
- iii. indicate whether the diagnosis was easy or difficult.

2.4 Ethics statement

This study was conducted in accordance with the guidelines of the Dutch Federation of Biomedical Scientific Societies (www.federa.org/codes-conduct), which state that no separate ethical approval is required for the use of anonymized residual tissue procured during regular treatment.

2.5 Statistical analysis

Data were analyzed after all participants had completed their assessments, using R Core Team (2020) (Version 4.0.0, <https://www.R-project.org/>). Histological diagnoses and features were assessed categorically. Inter-observer agreement

was measured by computing (i) percentages of agreement – to obtain an absolute measure, and (ii) kappa (κ) statistics – to obtain a relative measure. Fleiss' κ was computed to measure the overall agreement, i.e. agreement among all participants, using packages 'irr' and 'raters' [27, 28]. Cohen's κ was computed to measure the agreement between each participant-pair; this resulted in 36 κ -values for the diagnoses, as well as for each of the 15 histological features. Cohen's κ was also used to measure the concordance of the p53-IHC patterns with the consensus diagnoses. Bootstrapping (10,000 runs) was performed to calculate the 95% confidence intervals (CI) of the κ -values using the package 'boot' [29]. κ -values were interpreted as follows: < 0.20 = slight, $0.21 - 0.40$ = fair, $0.41 - 0.60$ = moderate, $0.61 - 0.80$ = substantial, or $0.81 - 1.00$ = near-perfect agreement. Correlation between categorical variables was measured with Chi (χ^2)-squared test; two-sided p-value < 0.05 was considered statistically significant. Heat-maps and bar-charts were constructed to visualize the data.

3. Results

3.1 Participants

Nine pathologists participated in this study; 6 practice at 5 non-academic centers in the Netherlands, which handle a high diagnostic case-load, and 3 practice at 2 academic centers in Belgium. Lengths of their practice experience ranged from less than 5 years ($n = 2$) to more than 15 years ($n = 3$). All participants routinely read vulvar pathology cases, including dVIN and VSCC, in their practice. The participants have been anonymized and are represented by acronyms (P1 – P9), which do not correspond to their order in the author-list.

3.2 Histological assessment

The participants diagnosed 28 – 81% (median = 58%) of the slides as dVIN, and perceived 21 – 80% (median = 58%) of these diagnoses to be difficult [Figure 1]. Nineteen to seventy-two percent (median = 42%) of the slides were diagnosed as no-dysplasia, of which 6 – 73% (median = 29%) were perceived as difficult.

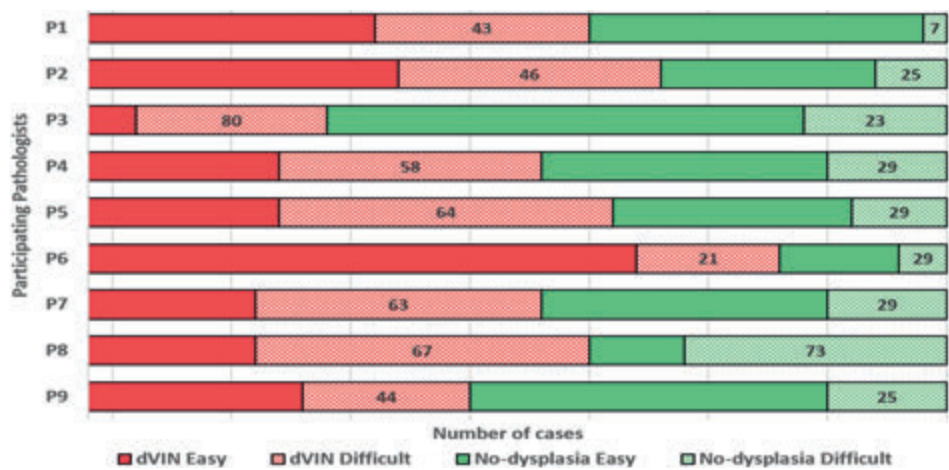


Figure 1: Bar-chart depicting the proportions of slides diagnosed as dVIN or no-dysplasia per participant (P1 – P9), along with the proportion of slides perceived as diagnostically difficult; the numbers depict the percentages of slides perceived as difficult

3.2.1 Consensus diagnoses and diagnostic agreement for dVIN

The consensus diagnosis for 23 slides (64%) was dVIN [Figure 2]. For these slides, rates of diagnostic agreement ranged from 56 – 100% (median = 78%). Unanimous agreement (100%) was obtained for 5 slides. The consensus diagnosis for 13 (36%) slides was no-dysplasia [Figure 2]. For these slides, rates of diagnostic agreement ranged from 67 – 100% (median = 89%). Unanimous agreement was obtained for 4 slides.

The overall agreement for the diagnosis of dVIN was moderate ($\kappa = 0.42$), and the pair-wise agreements ranged from slight ($\kappa = 0.10$) to substantial ($\kappa = 0.73$) [Table 1]. Substantial agreement was obtained between 19%, and moderate agreement between 39% of the participant-pairs [Figure 2]. Pair-wise κ -values with 95% CI are provided in Table S1. The diagnosis of dVIN was more frequently perceived to be difficult than the diagnosis of no-dysplasia ($p = 0.02$). For all slides (dVIN or no-dysplasia), diagnostic difficulty perceived by the participants correlated significantly with lower percentages of agreement ($p = 0.001$).

3.2.2 Correlation of the consensus diagnoses with p53-IHC patterns

Of the slides with a consensus diagnosis of dVIN, 17 (74%) showed p53-mutant patterns, which were basal to parabasal / diffuse overexpression in 15 slides, basal overexpression in 1 slide, and null-pattern in 1 slide. Six slides (26%) showed p53-wild-type pattern, i.e. scattered, heterogeneous, basal/parabasal expression [Figure 2].

All slides with a consensus diagnosis of no-dysplasia showed p53-wild-type pattern, i.e. scattered, heterogeneous, basal / parabasal expression [Figure 2].

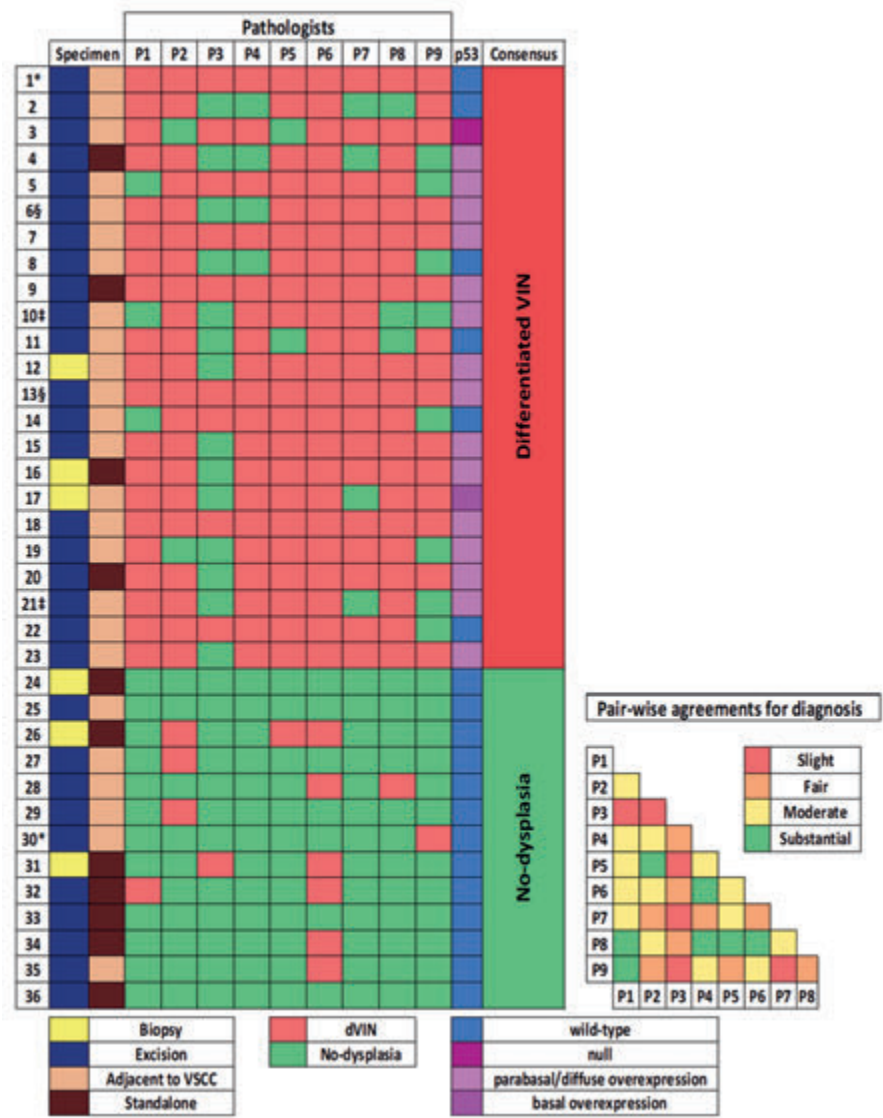


Figure 2: Left: Heat-map depicting the types of specimens, diagnoses rendered by the participants (P1-P9), p53-IHC results, and the consensus diagnoses; *, ‡, § slides were from the same specimen; Right: Heat-map depicting the levels of agreement between the participant-pairs for the diagnosis; color-coding corresponds to the levels of agreement

Concordance of the p53-IHC patterns with the consensus diagnoses was substantial ($\kappa = 0.67$; $p < 0.001$).

3.2.3 Agreements in the interpretation of histological features and ratings of their usefulness

Overall agreement was moderate in the interpretation of parakeratosis, mitotic count > 5/5 mm, and atypia discernable under 100X magnification. Fair agreement was obtained for multinucleation, angulated nuclei, chromatin abnormality, suprabasal mitoses, deep squamous eddies, elongated and / or anastomosing rete ridges, altered cellular alignment, individual cell keratinization, and cobblestone appearance [Table 1].

Pair-wise agreements in the interpretation of the histological features ranged from slight ($\kappa = 0.01$) to near-perfect ($\kappa = 0.94$) [Table 1]. The highest proportion of substantial / near-perfect agreement between participant-pairs was obtained for parakeratosis (39%), and cobblestone appearance was rated most frequently (24%) as ‘very useful’ for the diagnosis of dVIN [Table 2]. Taking into consideration the levels of pair-wise agreements and the ratings of usefulness, the most helpful features were – parakeratosis, cobblestone appearance, chromatin abnormality, angulated nuclei, atypia discernable under 100X, and altered cellular alignment.

For each histological feature, the levels of pair-wise agreements are depicted in Figures S1 and S2, and the pair-wise κ -values with 95% CI are provided in Tables S2 – S16. The ratings of usefulness are depicted in Figure 3, and the histological features are demonstrated in Figures 4 and 5.

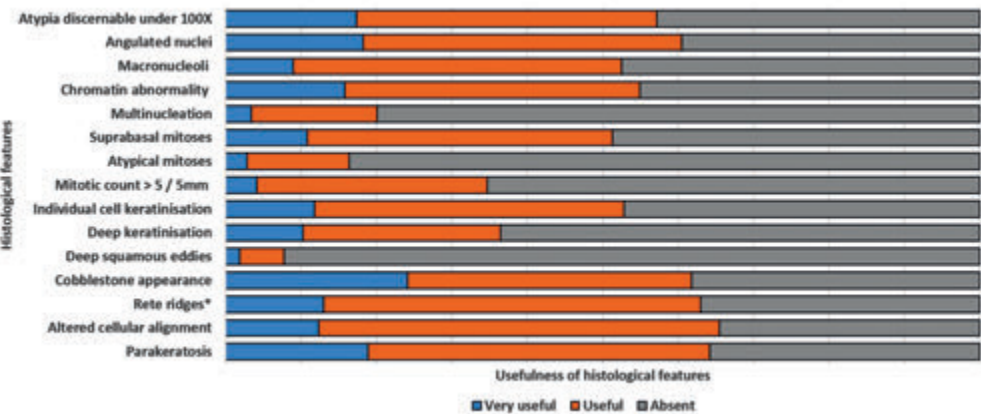


Figure 3: Bar charts representing the proportions of ratings of usefulness for each histological feature; *elongated and/or anastomosing rete ridges

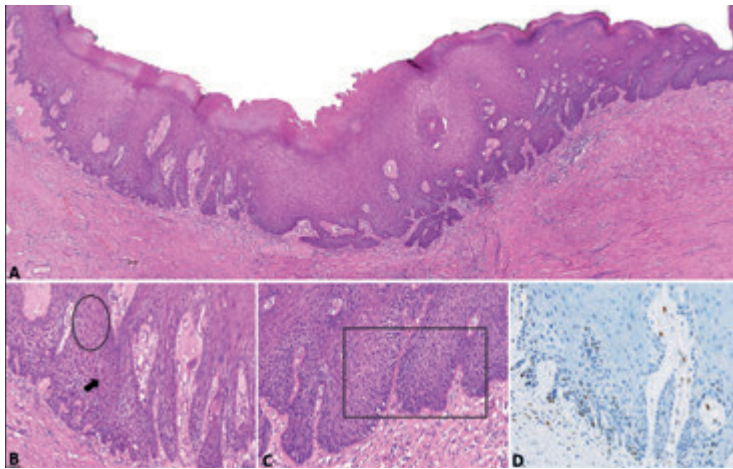


Figure 4: Low (A) and high magnification (B and C) images of one of the slides (HE-stain) that was diagnosed by all participants as dVIN, with corresponding p53-IHC (D) A. Epithelial acanthosis, hyperkeratosis, parakeratosis, and an eosinophilic appearance can be appreciated under low magnification (original magnification 5X). B. Cobblestone appearance (circled area), deep squamous eddies (arrow), elongated rete ridges, and C. altered cellular alignment and angulated nuclei (squared area) were rated by the participants as 'very useful' features for the diagnosis for this slide (original magnification 200X); D. p53-IHC shows a wild-type pattern, i.e. scattered nuclear p53 staining of heterogeneous intensity in the basal and the parabasal layers

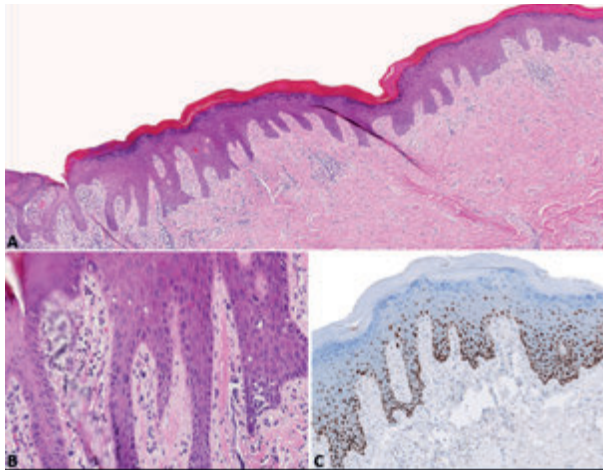


Figure 5: Low (A) and high magnification (B) images of one of the slides (HE-stain) that was diagnosed by 5 participants as dVIN and 4 participants as no-dysplasia, with corresponding p53-IHC (C) A. Elongated rete ridges and hyperkeratosis are appreciable under low magnification (original magnification 5X). B. Participants who diagnosed this slide as dVIN rated angulated nuclei, chromatin abnormality, cobblestone appearance, elongated rete ridges, and altered cellular alignment as 'very useful' features for the diagnosis (original magnification 300X); C. p53-IHC shows mutant pattern, i.e. diffuse, strong, nuclear p53 staining in the basal and parabasal layers

Table 1: Kappa values for the histological diagnoses and features of dVIN

	Overall agreement		Pair-wise agreements
	κ -values (95% CI)	Level of agreement	Range of κ -values
Histological diagnosis	0.42 (0.40 – 0.49)	Moderate	0.10 – 0.73
Histological features			
<i>Nuclear atypia</i>			
Atypia discernable under 100X magnification	0.42 (0.33 – 0.46)	Moderate	0.06 – 0.70
Angulated nuclei	0.32 (0.29 – 0.35)	Fair	0.01 – 0.65
Macronucleoli	0.10 (0.04 – 0.13)	Slight	0.01 – 0.68
Chromatin abnormality	0.32 (0.27 – 0.37)	Fair	0.08 – 0.67
Multinucleation	0.34 (0.32 – 0.41)	Fair	0.01 – 0.87
Suprabasal mitoses	0.28 (0.22 – 0.31)	Fair	0.01 – 0.61
Atypical mitoses	0.11 (0.04 – 0.14)	Slight	0.01 – 0.91
Mitotic count > 5 / 5mm	0.45 (0.38 – 0.51)	Moderate	0.01 – 0.94
<i>Disturbed maturation and architecture</i>			
Individual cell keratinization	0.21 (0.17 – 0.24)	Fair	0.01 – 0.46
Deep keratinization	0.19 (0.13 – 0.21)	Slight	0.01 – 0.48
Deep squamous eddies	0.31 (0.23 – 0.42)	Fair	0.06 – 0.60
Cobblestone appearance	0.22 (0.19 – 0.27)	Fair	0.10 – 0.61
Elongated and / or anastomosing rete ridges	0.30 (0.22 – 0.35)	Fair	0.04 – 0.69
Altered cellular alignment	0.23 (0.18 – 0.29)	Fair	0.01 – 0.73
Parakeratosis	0.57 (0.49 – 0.61)	Moderate	0.17 – 0.82

Table 2: Histological features of dVIN, in descending order of the proportions of substantial / almost-perfect agreement, and ratings as 'very useful' for diagnosis

Proportion of substantial / near-perfect agreement	Very useful for the diagnosis of dVIN
Parakeratosis (39%)	Cobblestone appearance (24%)
Mitotic count > 5/5 mm (19%)	Parakeratosis (19%)
Deep squamous eddies (14%)	Angulated nuclei (18%)
Multinucleation (11%)	Atypia discernable under 100X (16%)
Chromatin abnormality (8%)	Chromatin abnormality (16%)
Atypical mitoses (8%)	Elongated and / or anastomosing rete ridges (13%)
Atypia discernable under 100X (6%)	Altered cellular alignment (12%)
Angulated nuclei (6%)	Individual cell keratinization (12%)
Macronucleoli (6%)	Suprabasal mitoses (11%)
Cobblestone appearance (6%)	Deep keratinization (10%)
Altered cellular alignment (6%)	Macronucleoli (9%)
Elongated and / or anastomosing rete ridges (5%)	Multinucleation (3%)
Suprabasal mitoses (3%)	Mitotic count > 5 / 5mm (3%)
Individual cell keratinization (0%)	Atypical mitoses (3%)
Deep keratinization (0%)	Deep squamous eddies (2%)

4. Discussion

To the best of our knowledge, this is the first bi-national, multi-institutional, ring-study to assess the inter-observer agreement in the histological assessment of dVIN. Agreement on the diagnosis between nine participating pathologists was moderate, while that between the participant-pairs varied from slight to substantial. These results were similar to that of the only previous study on inter-observer agreement in dVIN [13], and indicates that the diagnostic agreement for dVIN remains suboptimal.

As histological diagnoses guide treatment decisions, variability in the diagnoses can result in treatment disparities [31]. Therefore, to improve the diagnostic reliability and to assure a similar standard of care, we suggest consensus evaluation of dVIN cases with a panel of pathologists experienced in vulvar neoplasia. Regular inter-disciplinary communication between gynecologists / dermatologists and pathologists can also enhance relevant knowledge and expertise.

An essential step to ensure a reliable histological diagnosis is to identify representative features which can be reproducibly interpreted by pathologists. We identified the most helpful features as parakeratosis, cobblestone appearance, chromatin abnormality, angulated nuclei, atypia discernable under 100X, and altered cellular alignment, based on the proportions of substantial / near-perfect agreement between the participant-pairs, and the ratings of diagnostic usefulness. We observed that the participants recorded parakeratosis and cobblestone appearance as very useful for diagnosing dVIN, particularly where the nuclear atypia could not be discerned under 100X.

Previously, van den Einden et al. proposed that the presence of atypical mitoses in the basal layer, basal cellular atypia, dyskeratosis, prominent nucleoli, and elongated and anastomosing rete ridges were the most predictive features of dVIN [13]. In a subsequent survey among vulva pathology experts, only basal layer atypia was judged by consensus as an 'essential' diagnostic feature [14]. However, neither of these studies assessed the agreement in the interpretation of these features. In our previous study, we obtained substantial agreement in the interpretation of macronucleoli, angulated nuclei, individual cell keratinization, deep keratinization, and deep squamous eddies, between pathologists at our center [15]. In the current study, however, similar level of agreement for these features was not observed. We speculate that our previous results may have

been influenced by the similar standard of histological interpretation among participants who work in close collaboration at the same center.

In this study, we also correlated the histological consensus diagnoses with the immunohistochemical expression of p53, as this marker is commonly used to aid the diagnosis of dVIN. p53-mutant patterns have been reported to accurately reflect underlying *TP53* mutations, which characterize dVIN [19, 20, 32]. Substantial concordance of p53-IHC patterns with the histological consensus diagnoses was recorded, which confirms that routine use of this marker can improve the diagnostic accuracy for dVIN.

However, 6 (26%) of the slides in this study that were diagnosed as dVIN by consensus, showed wild-type p53-expression. This is in line with recent literature, which states that 17 – 42% cases of dVIN can show wild-type p53-expression [4], and implies that p53-IHC may not effectively inform the diagnosis in every case of dVIN. Furthermore, p53-IHC patterns in VSCC and the adjacent dVIN may not show perfect concordance [22]. A recent study reported that while dVIN adjacent to p53-wild type VSCC always shows wild-type p53-expression, dVIN adjacent to p53-mutant VSCC can show wild-type p53-expression in 31.4% of cases [22]. In our study, all of the lesions judged as dVIN by consensus and showing wild-type p53-expression were present adjacent to VSCC. Similarly to the previous study [22], we observed that 67% (4/6) of these VSCCs showed wild-type p53-expression, while 33% (2/6) showed p53-mutant patterns (results not presented). This limitation of p53-IHC should be borne in mind particularly when using this marker to confirm the presence of dVIN in resection margins of VSCC. For dVINs that show wild-type p53-expression, the diagnosis defers to histological assessment, which, as our study indicates, may be fraught with variability. In view of this, we believe that ancillary biomarkers (immunohistochemical / molecular) need to be established to aid the diagnosis of the p53-wild-type subcategory of dVIN.

Through this study, we intended to estimate the diagnostic variability of dVIN in the real-world. To ensure an accurate representation of this variability, (i) pathologists with varying levels of experience, and from academic and non-academic centers were included, (ii) diagnostic criteria were not pre-determined to allow the participants to interpret the histology in light of their own experience, and, (iii) assessments of outlier participants were not excluded.

Nevertheless, there are several limitations of this study. We used the majority (consensus) diagnosis of each slide to determine the diagnostic gold-standard. It could be argued whether the consensus represents another diagnostic opinion rather than a standard of truth. dVIN is known to originate in a background of chronic dermatoses, and there is no clear, universally-accepted threshold for identifying atypia / dysplasia. This threshold is often influenced by the pathologists' training and / or practice experience. Unless a reliable IHC-marker is established, every method to ascertain a gold-standard diagnosis will have some bias.

There is also little consensus on the ideal method for measuring observer agreement in pathology diagnosis. It has been suggested that both percentages of agreement and κ -statistics do not take into account the prevalence of a particular diagnosis in a set of cases, or completely rule out concordances due to chance [33, 34]. Validity of the cut-offs that are used to interpret levels of agreement from κ -values has also been challenged [30, 35].

It could also be argued whether our study over-estimated the diagnostic variability. Unlike in routine practice, participants diagnosed the slides without clinical information, serial sections, or IHC. The selection contained a higher proportion of dVIN than no-dysplasia slides, which may not reflect routine practice. We lacked statistical power to evaluate the influence of level of experience or practice setting on the diagnostic variability. Furthermore, the inter-observer agreement in the interpretation of p53-IHC was not assessed. To gain further insights on these contexts, we have set up a larger study among geographically disparate group of pathologists, which includes the assessment of p53-IHC.

5. Conclusion

In conclusion, the suboptimal level of diagnostic agreement for dVIN observed in this study affirms the difficulty of the diagnosis. We identified parakeratosis, cobblestone appearance, chromatin abnormality, angulated nuclei, atypia discernable under 100X, and altered cellular alignment as helpful diagnostic features of dVIN. For cases with a histological suspicion of dVIN, we suggest consensus-based pathological evaluation to improve diagnostic reliability.

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6



CHAPTER 6

EVALUATION OF IMMUNOHISTOCHEMICAL MARKERS CK17 AND SOX2, AS ADJUNCTS TO P53, FOR THE DIAGNOSIS OF DIFFERENTIATED VULVAR INTRAEPITHELIAL NEOPLASIA

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Abstract

Histological diagnosis of differentiated vulvar intraepithelial neoplasia (dVIN), the precursor of human papillomavirus (HPV)-independent vulvar squamous cell carcinoma (VSCC), can be challenging, as features of dVIN may mimic those of non-dysplastic dermatoses. To aid the diagnosis, p53-immunohistochemistry (IHC) is commonly used, and mutant expression patterns are used to support a histological diagnosis of dVIN. However, a proportion of dVIN can show wild-type p53-expression, which is characteristic of non-dysplastic dermatoses. Furthermore, recent research has identified a novel precursor of HPV-independent VSCC – the p53-wild-type differentiated exophytic vulvar intraepithelial lesion (de-VIL). Currently, there are no established diagnostic IHC-markers for p53-wild-type dVIN or de-VIL.

We evaluated IHC-markers, cytokeratin 17 (CK17) and SRY-box 2 (SOX2), as diagnostic adjuncts for dVIN. For this, IHC-expression of CK17, SOX2, and p53 was studied in dVIN (n = 56), de-VIL (n = 8), and non-dysplastic vulvar tissues (n = 46). For CK17 and SOX2, the percentage of cells showing expression, and the intensity and distribution of expression were recorded. We also performed next generation targeted sequencing (NGTS) on a subset of dVIN (n = 8) and de-VIL (n = 8).

With p53-IHC, 74% of dVIN showed mutant patterns and 26% showed wild-type expression. Median percentage of cells expressing CK17 or SOX2 was significantly higher in dVIN (p53-mutant or p53-wild-type) and de-VIL, than in non-dysplastic tissues ($p < 0.01$). Diffuse, moderate-to-strong, full epithelial expression of CK17 or SOX2 was highly specific for dVIN and de-VIL. With NGTS, *TP53* mutations were detected in both dVIN and de-VIL.

We infer that immunohistochemical markers CK17 and SOX2, when used along with p53, may help support the histological diagnosis of dVIN.

1. Introduction

Vulvar squamous cell carcinoma (VSCC) is classified etiologically into human papilloma virus (HPV)-related, and HPV-independent subtypes [1-4]. HPV-independent VSCC is the more prevalent subtype [3] that typically arises in the setting of chronic dermatoses. Differentiated vulvar intraepithelial neoplasia (dVIN) is the most well-characterized precursor lesion of HPV-independent VSCC [1-4]. Studies report that dVIN can progress rapidly to VSCC [5,6], therefore, lesions diagnosed on histology as dVIN are surgically excised [7,8]. However, histological diagnosis of dVIN can be difficult even for experienced pathologists [2], and may suffer from suboptimal reproducibility [9,10].

The difficulty in diagnosing dVIN stems largely from its subtle histological appearance, which may mimic that of reactive / non-dysplastic dermatoses [11-14]. Therefore, to accurately discriminate dVIN from non-dysplastic dermatoses, p53-immunohistochemistry (IHC) is commonly used as an ancillary tool [12,13,15]. Mutant patterns of p53-expression are used to support a histological diagnosis of dVIN, as these have been reported to reflect *TP53* mutations that characterize dVIN [16-18]. However, 17 – 42% of dVIN can show wild-type p53-expression, which is usually observed in non-dysplastic lesions [19-22]. Furthermore, recent research has identified a novel putative precursor of HPV-independent VSCC, named differentiated exophytic vulvar intraepithelial lesion (de-VIL) [23]. de-VILs are acanthotic or verruciform lesions lacking sufficient histological atypia for the diagnosis of dVIN, and on IHC, show wild-type p53-expression [23-25]. There is a need for IHC markers that may facilitate the diagnosis of precursors of HPV-independent VSCC, in particular, the p53-wild-type lesions [17,26].

In recent years, multiple studies have investigated immunohistochemical expression of cytokeratin 17 (CK17) and SRY-box transcription factor 2 (SOX2), in precursors of genital and non-genital SCCs [20,27-35]. CK17 is a differentiation marker that maintains cellular organization [36], and SOX2 is a stemness regulator that maintains self-renewal properties of normal and malignant cells [36]. CK17 and SOX2 have been reported to show increased expression in SCC precursors compared to normal tissues, and both have been deemed as promising diagnostic markers [20,27,33-35].

In this study, we evaluated immunohistochemical markers CK17 and SOX2 as adjuncts to p53 for the diagnosis of dVIN. For this, we examined the expression of these markers in dVIN, de-VIL, high-grade squamous intraepithelial lesion (HSIL;

precursor of HPV-related VSCC), and non-dysplastic vulvar tissue. In addition, to facilitate the identification of other potential diagnostic markers, we performed next generation targeted sequencing (NGTS) on a subset of dVIN and de-VIL.

2. Materials and Methods

This retrospective, observational study follows the Standards for Reporting Diagnostic Accuracy (STARD) guidelines [37]; the checklist is provided in supplement 1.

2.1 Histology review

Vulvar lesions from 2014 – 2017 were identified from the electronic records of the Department of Pathology, Erasmus MC, and their hematoxylin-eosin (HE) stained glass slides were retrieved from the archives. These slides were reviewed by two pathologists (SDG and PCEG), and the lesions were classified as VSCC, dVIN, HSIL, de-VIL, or non-dysplastic vulvar tissue, using diagnostic criteria from literature [3,9,11-13,20,23,24,38-40]. Clinical data (age and treatment information) were gathered from the patient records. All patient data were anonymized and patient materials were handled following the guidelines of World Medical Association Declaration of Helsinki.

2.2 Immunohistochemistry (IHC)

For IHC, a set of dVIN, HSIL, de-VIL, and non-dysplastic vulvar cases having sufficient tissue to prepare serial sections was selected. To ensure a comprehensive study of the IHC-expression, the selection was prepared in a way to include – (i) lesions on vulvar skin with or without adnexal structures; (ii) standalone lesions, and lesions in tissue adjacent to VSCC; and (iii) lesions representing a range of histological appearances for each diagnosis. Cases with a history of chemo/radiotherapy were excluded.

Formalin-fixed paraffin embedded (FFPE)-tissues of the selected lesions were retrieved from the archives, and 4µm-thick serial sections were prepared. For all lesions, IHC with p53 (Bp53-11), p16 (CintecR®), dual stain CK17-MIB1 (Sp95-Ki67), and SOX2 (Sp67) was performed using an automated and validated staining system (Ventana Benchmark ULTRA, Ventana Medical Systems, Tucson, AZ, USA). The IHC protocol is detailed in supplement 2.

For each lesion, the areas most representative of the diagnosis were marked on the HE-slides, so that identical areas could be studied on the IHC-slides. IHC was scored by SDG and PCEG by visual estimation, as described below.

p16: block-type expression = continuous, strong, nuclear and / or cytoplasmic expression involving $\geq 1/3$ rd of epithelial thickness [41]; non-block type expression = patchy expression in clusters of cells; no expression = complete lack of expression.

p53: mutant patterns = diffuse (basal to parabasal) overexpression / basal overexpression / null-pattern / cytoplasmic expression; wild-type pattern (scattered) = scattered, heterogeneous, basal or parabasal expression; wild-type pattern (mid-epithelial) = heterogeneous, mid-epithelial expression with sparing of basal cells and / or lower parabasal cells [16-18].

MIB1: increased expression = increased nuclear expression in the basal and / or parabasal layers; not increased expression = sporadic nuclear expression in basal (minor component) layers and / or parabasal layers (major component) [30,39].

CK17 and SOX2: The following parameters were recorded – (i) percentage of cells showing uniform cytoplasmic (CK17) or nuclear (SOX2) expression, irrespective of the intensity (ii) intensity of expression (weak / moderate / strong), and (iii) distribution of expression within the epithelium. In case of variation in the intensity and / or distribution, the predominant pattern was scored.

2.3 Next generation targeted sequencing (NGTS)

NGTS was performed on a subset of dVIN and all de-VIL. The methodology is detailed in supplement 3. In brief, areas having minimum 50% lesional cells were selected, and micro-dissected manually from hematoxylin-stained slides into 5% Chelex 100 resin (BioRad Laboratories, Hercules, CA) cell lysis solution (Promega, Madison, WI, USA). Tissue fragments were subjected to proteinase K digestion for 16 hours at 56 °C. After inactivating proteinase K, and removing cell debris and Chelex resin by centrifugation, the extracted DNA was used without further purification. DNA-concentration was measured with Qubit 2.0 fluorometer (Thermo FisherScientific, Waltham, MA, USA).

For NGTS, a custom-made panel, designed using the AmpliSeq designer (Thermo Fisher Scientific, Waltham, MA) was used. This panel comprises 1042 amplicons covering hotspot regions in 65 cancer-related genes, and single nucleotide polymorphisms [listed in supplement 3]. NGTS was performed using the Ion Torrent platform (Thermo Fisher Scientific) following manufacturer's protocols. Ion AmpliSeq Library Kitplus-384 LV was used for library preparation.

Sequence information was analyzed with Variant Caller v.5.10.0.18 (ThermoFisher Scientific), and variants were annotated in a local Galaxy pipeline using ANNOVAR (version 2014-11-12, Wang Genomics Lab, University of Southern California, Los Angeles, CA). For reporting, variants existing in Exome Variant Server NHLBI GO Exome sequencing project (ESP), the 100,000 Genomes Project, Genome Aggregation Database (GnomAD), or Genome of the NL (GoNL) databases were filtered out, and those having a variant allele frequency > 10%, and located in the exons or splice sites were included (with the exception of *TERT*-promoter mutations). Pathogenicity of coding non-synonymous variants were predicted using Protein Variation Effect Analyzer (PROVEAN), Sorting Tolerant From Intolerant (SIFT), and UMD-Predictor algorithms, and the Catalogue of Somatic Mutations in Cancer (COSMIC).

2.4 Ethics statement

This study follows the guidelines of the Dutch Federation of Biomedical Scientific Societies (www.federa.org/codes-conduct), which state that no separate ethical approval is required for the use of anonymized residual tissue procured during regular treatment.

2.5 Statistical analyses

Data were analyzed using SPSS Statistics (Version 25.0. Armonk, NY: IBM Corp). Independent sample's t-test was used to compute two-sided p-values and identify statistically significant differences. p-value < 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curves were plotted for p53, CK17, and SOX2, and areas under the curve (AUC) with 95% confidence intervals (CI) were measured, to assess their individual sensitivity and specificity for the diagnosis of dVIN. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of p53, CK17, and SOX2 for the diagnosis of dVIN were computed, using the histological diagnoses as gold-standard. Expression levels of CK17 and SOX2 were visualized by constructing bar-charts and box-plots. NGTS data were analyzed descriptively.

3. Results

3.1 Histology review

A total of 137 lesions were included after histology review, comprising dVIN (n = 56), de-VIL (n = 8), HSIL (n = 27), and non-dysplastic vulvar tissue (n = 46). Non-dysplastic tissues comprised lichen sclerosus (n = 15), non-specific reactive changes / inflammation (n = 18), and histologically normal vulvar tissue (n = 13). Six of the histologically normal vulvar tissues were identified from resection specimens of VSCC, and 7 were residual tissues from vulvar surgeries conducted for benign pathologies in women with no history of VIN or VSCC. Clinico-pathological characteristics are presented in Table 1.

3.2 Immunohistochemistry (IHC)

Immunohistochemistry results are presented in Table 2, illustrated in Figures 1 and 2, and summarized below.

p16: Of HSIL, 96% showed block-type, and 4% showed non-block-type expression. Block-type expression is considered to be a reliable surrogate marker of high-risk HPV-infection [41], and this was not seen in any dVIN, de-VIL, or non-dysplastic vulvar tissues.

p53: Of dVIN, 74% showed mutant patterns, and 26% showed wild-type (scattered) expression. All de-VIL showed wild-type (scattered) expression. Of HSIL, 96% showed wild-type (mid-epithelial) expression; this included the lesion that showed non-block-type p16-expression. Of non-dysplastic vulvar tissue, 83% showed wild-type (scattered), and 17% showed mutant patterns of expression.

MIB1: Increased MIB1 expression was noted in 52% of dVIN, 88% of de-VIL, all HSILs, and 39% of non-dysplastic vulvar tissues. For dVIN, de-VIL, and non-dysplastic lesions, increased MIB1 expression was most frequently observed in the basal and / or parabasal layers, whereas, for HSIL, this was across full epithelial thickness. Difference in MIB1 expression in dVIN and non-dysplastic vulvar tissues was not significant ($p = 0.08$).

CK17: For dVIN and de-VIL, median percentage of cells expressing CK17 was significantly higher ($p < 0.01$) compared to non-dysplastic vulvar tissues [Figures 1 and 2]. Increased CK17-expression was recorded for

dVIN that showed mutant pattern or wild-type p53-expression, with no significant difference among these sub-groups, in comparison with non-dysplastic vulvar tissue.

Of dVIN, 80% showed diffuse, moderate-to-strong expression, either across full epithelial thickness or in the suprabasal layers. This pattern of expression was also noted in 88% of de-VIL, and 63% of HSIL. In dVIN, de-VIL, and HSIL, expression of CK17 followed the distribution of dysplastic cells. Of non-dysplastic vulvar tissue, 80% showed either no expression, or patchy, weak, expression in the suprabasal layers.

SOX2: For dVIN and de-VIL, median percentage of cells expressing SOX2 was significantly higher ($p < 0.01$) compared to non-dysplastic vulvar tissue [Figures 1 and 2]. Increased SOX2-expression was recorded for dVIN, that showed mutant pattern or wild-type p53-expression, with no significant difference among these sub-groups, in comparison with non-dysplastic vulvar tissue.

Of dVIN, 86% showed diffuse, moderate-to-strong expression, either across full epithelial thickness or in the basal and suprabasal layers. This pattern of expression was also noted in 88% of de-VIL, and 88% of HSIL. In dVIN, de-VIL, and HSIL, expression of SOX2 followed the distribution of dysplastic cells. Of non-dysplastic vulvar tissue, 81% showed either no expression, or scattered, weak, expression in the basal (predominantly) and suprabasal layers.

Table 1: Clinico-pathological characteristics

	Mean age (95% CI)	Adjacent*	Standalone	Vulvar skin with adnexa	Vulvar skin without adnexa
	Number of cases (percentage)				
dVIN (n = 56)	69.3 (66.0 – 72.8)	35 (63)	21 (38)	37 (66)	19 (34)
de-VIL (n = 8)	68.3 (58.9 – 77.6)	4 (50)	4 (50)	6 (75)	2 (25)
HSIL (n = 27)	64.2 (57.8 – 70.5)	8 (30)	19 (70)	24 (89)	3 (11)
Non-dysplastic vulvar tissue (n = 46)	63.2 (58.2 – 68.2)	23 (50)	23 (50)	38 (83)	8 (17)

dVIN: differentiated vulvar intraepithelial neoplasia; de-VIL: differentiated exophytic vulvar intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion; *Adjacent to VSCC

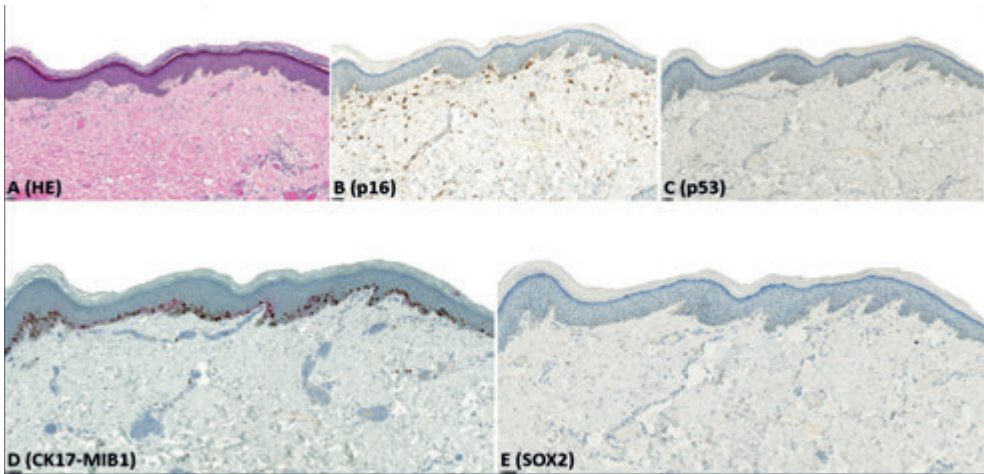


Figure 1: Example of immunohistochemical expression of p16, p53, CK17, MIB1, and SOX2 in lichen sclerosus (LS); A. Histological appearance of LS [hematoxylin-eosin (HE) stain]; B. complete lack of p16-expression; C. wild-type (scattered) p53-expression; D. focal, weak, CK17-expression and increased MIB1-expression E. complete lack of SOX2-expression.

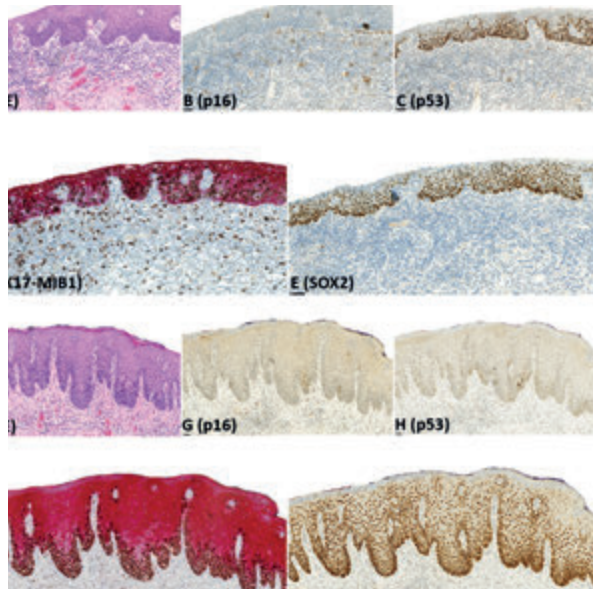


Figure 2: Example of immunohistochemical expression of p16, p53, CK17, MIB1, and SOX2 in differentiated vulvar intraepithelial neoplasia (dVIN); A. Histological appearance of dVIN (HE-stain); B. complete lack of p16-expression; C. mutant pattern (basal and parabasal overexpression) of p53-expression; D. diffuse, strong CK17-expression across full epithelial thickness and increased MIB1-expression; E. diffuse, strong SOX2-expression across full epithelial thickness; F. Histological appearance of dVIN (HE-stain); G. complete lack of p16-expression; H. wild-type (scattered) p53-expression; I. diffuse, strong CK17-expression across full epithelial thickness and increased MIB1-expression; J. diffuse, strong SOX2-expression across full epithelial thickness.

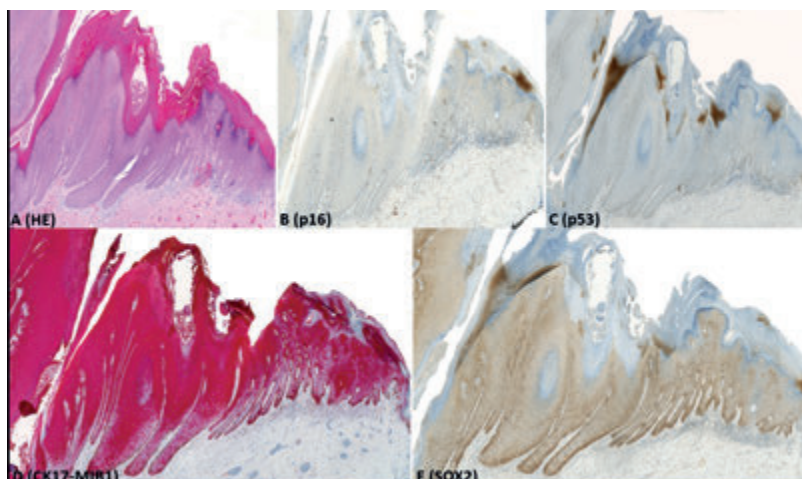


Figure 3: Example of immunohistochemical expression of p16, p53, CK17-MIB1, and SOX2 in differentiated exophytic vulvar intra-epithelial lesion (de-VIL); A. Histological appearance of de-VIL (HE-stain); B. complete lack of p16-expression; C. wild-type (scattered) p53-expression; D. diffuse, strong CK17-expression across full epithelial thickness and increased MIB1-expression; E. diffuse, strong SOX2-expression across full epithelial thickness.

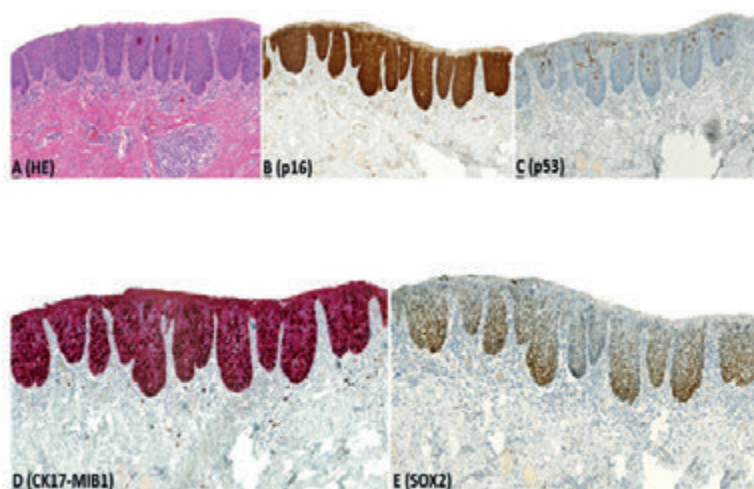


Figure 4: Example of immunohistochemical expression of p16, p53, CK17-MIB1, and SOX2 in high grade squamous intraepithelial lesion (HSIL); A. Histological appearance of HSIL (HE-stain); B. block-type p16-expression; C. wild-type (mid-epithelial) p53-expression; D. diffuse, strong CK17-expression across full epithelial thickness and increased MIB1-expression; E. diffuse, strong SOX2-expression across full epithelial thickness.

3.3 Receiver operating characteristic curve (ROC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV)

For the diagnosis of dVIN, area under the curve (AUC) for p53 was 0.78 (95% CI: 0.69 – 0.87); for CK17, this was 0.82 (95% CI: 0.74 – 0.91), and for SOX2, this was 0.87 (95% CI: 0.79 – 0.94) [Figure 3]. SOX2 showed the highest sensitivity (86%; 95% CI: 77.6 – 92.1%) for the diagnosis of dVIN, whereas p53 showed the highest specificity (83%; 95% CI: 73.4 – 89.5%). SOX2 also showed the highest PPV (82%; 95%CI: 74.9 – 87.2%) and NPV (85%; 95% CI: 77.9 – 90.5%) for the diagnosis of dVIN [detailed in supplement 4].

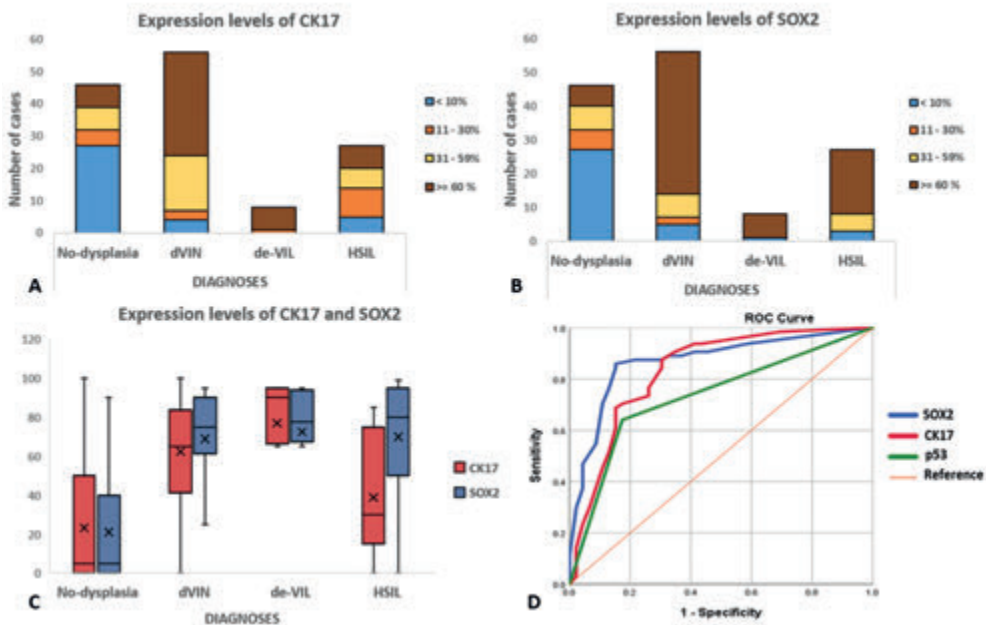


Figure 5: A. Bar-charts depicting the levels of CK17-expression and B. SOX2-expression. For each diagnostic category; color-coding represents the percentage of cells showing cytoplasmic CK17-expression, or nuclear SOX2-expression stratified as < 10%, 11 – 30%, 31 – 59%, and ≥ 60%; C. Boxplots depicting the distribution of CK17 and SOX2 expression for each diagnostic category; horizontal lines in the boxes represent the medians, the cross-marks represent the mean, and the whiskers represent the 5th and 95th percentiles; D. ROC curves for SOX2 (blue line), CK17 (red line), and p53 (green line) immunohisto-chemistry for the diagnosis of dVIN. Area under the curve (AUC) for SOX2 = 0.87, CK17 = 0.82, and p53 = 0.78.

3.4 Next generation targeted sequencing

In the subset of dVIN (n = 8) and de-VIL (n = 8) that was studied, a total of 106 pathogenic mutations in 44 genes were identified [supplement 5]. Median number of mutations detected in dVIN was 3 (range: 1 – 6), and in de-VIL was 5 (range: 1 – 31). Pathogenic mutations detected in at least 3 samples of dVIN or de-VIL are depicted in Figure 4. The most commonly detected mutations for both diagnoses are described below.

dVIN: For all dVIN, mutations in *TP53* (63%), *TERT*-promoter (50%), *CDKN2A* (38%), *RNF43* (38%) were most frequently detected. For p53-wild-type dVIN (n = 4), these were *TP53* (50%) and *CDKN2A* (50%), while for p53-mutant dVIN (n = 4), these were *TP53* (75%) and *RNF43* (50%).

de-VIL: For de-VIL, *RNF43* (63%), *TP53* (63%), *PIK3CA* (50%), *ARID1A* (50%), and *KEAP1* (50%) were most frequently detected. Mutations in *KEAP1* (50%), *CDH1* (38%), *PIK3R1* (38%), and *POLE* (38%) were exclusively detected in de-VIL, i.e. not detected in dVIN.

Table 2: Immunohistochemical expression of p53, p16, CK17, SOX2, and MIB1

Immunohistochemical marker		Expression patterns	Diagnoses			
			dVIN (n = 56)	de-VIL (n = 8)	HSIL (n = 27)	Non-dysplastic vulvar tissue (n = 46)
			Number of cases (percentage)			
p53	mutant patterns	Parabasal / diffuse overexpression	30 (54)	0 (0)	0 (0)	1 (2)
		Basal overexpression	1 (2)	0 (0)	0 (0)	7 (15)
		Null-pattern	10 (18)	0 (0)	0 (0)	0 (0)
	wild-type patterns	Wild-type (scattered)	15 (26)	8 (100)	1 (4)	38 (83)
		Wild-type (mid-epithelial)	0 (0)	0 (0)	26 (96)	0 (0)
p16	Block-type expression	0 (0)	0 (0)	26 (96)	0 (0)	
	Non-block-type expression	2 (4)	0 (0)	1 (4)	7 (15)	
	No expression	54 (96)	8 (100)	0 (0)	39 (85)	
MIB1	Increased expression	29 (52)	7 (88)	27 (100)	18 (39)	
	Not increased expression	27 (48)	1 (12)	0 (0)	28 (61)	

Immunohistochemical marker	Expression patterns	Diagnoses			
		dVIN (n = 56)	de-VIL (n = 8)	HSIL (n = 27)	Non-dysplastic vulvar tissue (n = 46)
Number of cases (percentage)					
CK17	Diffuse, moderate-strong, full epithelial expression	27 (48)	5 (63)	6 (22)	1 (2)
	Diffuse, moderate-strong, suprabasal expression	18 (32)	2 (25)	11 (41)	3 (7)
	Patchy, moderate-strong, suprabasal expression	8 (14)	1 (12)	10 (37)	5 (11)
	Patchy, weak, suprabasal expression	2 (4)	0 (0)	0 (0)	19 (41)
	No expression	1 (2)	0 (0)	0 (0)	18 (39)
SOX2	Diffuse, moderate-strong, full epithelial expression	37 (66)	5 (63)	12 (44)	2 (4)
	Diffuse, moderate-strong, basal and suprabasal expression	11 (20)	2 (25)	12 (44)	7 (15)
	Scattered, weak, basal (predominant) and suprabasal expression	7 (13)	1 (12)	2 (8)	17 (37)
	No expression	1 (1)	0 (0)	1 (4)	20 (44)
	Median percentage of cells showing expression (95% CI)				
CK17		65 (55.3 – 69.4)	90 (53.7 – 95)	45 (27.6 – 50.2)	5 (14.4 – 32.1)
SOX2		75 (61.8 – 76.1)	78 (48 – 96.9)	80 (58.4 – 81.5)	5 (12.8 – 29.2)

dVIN: differentiated vulvar intraepithelial neoplasia; de-VIL: differentiated exophytic vulvar intraepithelial lesion; HSIL: high grade squamous intraepithelial lesion

4. Discussion

Since pathological diagnoses inform treatment decisions, accurate distinction of dVIN from reactive / non-dysplastic lesions is crucial. We observed that the majority of dVIN show diffuse, moderate-to-strong expression of CK17 and SOX2, across full epithelial thickness or in the suprabasal layers. In contrast, the majority of non-dysplastic lesions show either no expression, or patchy / scattered weak expression of CK17 and SOX2. We therefore infer that IHC with CK17 and SOX2 can help distinguish dVIN from non-dysplastic lesions.

Recent studies have increasingly recognized that accurate diagnosis of dVIN may not be achieved on the basis of histological assessment alone [42-45]. The spectrum of histological features of dVIN can overlap with that of non-dysplastic lesions, and occasionally, with that of HPV-related HSILs as well

[44,45]. Therefore, to improve diagnostic accuracy, an IHC panel comprising p16 and p53 is being recommended for lesions with a histological suspicion of dVIN [2,4,17,43]. Block-type p16-expression is a reliable surrogate of high-risk HPV-infection, and helps discriminate HSIL from dVIN [44,45]. In addition, mutant patterns of p53-expression, i.e. basal and / or parabasal over-expression, or complete absence of expression, can help distinguish dVIN from non-dysplastic lesions [46,47]. Furthermore, the mid-epithelial wild-type p53-expression, which is considered characteristic of HSIL (seen in 96% of HSIL in the current study), can also help discriminate HSIL from dVIN [48].

However, similarly to most IHC-markers, p53 is not without limitations. Increased p53-expression may not only result from the mutated protein, but also from the accumulation of the wild-type protein in response to DNA-damage in inflammatory conditions [46,49]. Therefore, increased p53-expression may be observed in non-dysplastic lesions as well [49]. Moreover, a proportion of dVIN (26% in our series), and the recently recognized VSCC precursor, de-VIL, show wild-type p53-expression [2,10,23,24]. In addition to p53 and p16, the proliferation marker MIB1 is commonly used to aid the diagnosis of dVIN. However, increased MIB1-expression can be seen both in dVIN and in reactive, non-dysplastic lesions [39,40].

Although over the past years, a number of novel diagnostic IHC-markers have been studied for dVIN, none have so far been translated to the clinics [15]. This is largely because most of these markers were evaluated in small cohorts and / or in single studies [15].

For dVIN, CK17 is the only IHC-marker that has been evaluated in more than one study, which includes one of our previous studies [20,27]. CK17 is a basal / myo-epithelial cell-associated protein [36]. In normal skin (genital / non-genital), CK17 is expressed only in the appendages, and not in the squamous epithelial lining [33,50]. Increased CK17-expression across full-epithelial thickness or in the suprabasal layers has been observed in SCC, and precursors of SCC, of the cervix [51], vulva [20,27], anus [33], larynx [34], and oral cavity [30].

SOX2 is another IHC-marker that has been widely studied in SCC of both genital and non-genital locations [28,29,32]. A recent meta-analysis identified SOX2 as a promising biomarker for tongue SCC, which is also an HPV-independent SCC [35]. SOX2 is a transcription factor postulated to promote cancer development and progression, and increased SOX2-expression has been observed in SCC,

and precursors of SCC, of the cervix [52], vulva [28,29], esophagus [53], and oral cavity [54].

To evaluate CK17 and SOX2 as diagnostic adjuncts for dVIN, we systematically examined their expression in a carefully selected set of dVIN, de-VIL, HSIL, and non-dysplastic vulvar tissues. Increased expression of CK17 was observed in dVIN (p53-mutant or p53-wild-type), compared to non-dysplastic vulvar tissue, in line with previous reports [20,27]. CK17-expression was also increased in de-VIL and HSIL, compared to non-dysplastic tissue. However, similarly to Podoll et al. [27], we also observed that CK17-expression can be higher in dVIN than in HSIL. This could be due to the paradoxical maturation exhibited by the dysplastic cells of dVIN, as it has been reported that CK17-expression is most prominent in dysplastic cells with some degree of phenotypic maturation [55].

For SOX2, increased expression was observed in dVIN (p53-mutant or p53-wild-type), compared to non-dysplastic vulvar tissue, in line with the previous report [29]. Increased SOX2-expression was also observed in de-VIL and HSIL, compared to non-dysplastic tissue. However, unlike CK17, SOX2-expression levels were fairly similar in dVIN and HSIL, as was also reported by Brustmann et al. [29].

For both CK17 and SOX2, we sought to identify lesion-specific expression patterns, as pattern-based IHC-interpretation can be more reproducible and easier to implement in research or practice, than manual counting of stained cells [30]. Diffuse, moderate-to-strong expression of CK17 or SOX2, across full epithelial thickness, or in the basal and suprabasal layers showed high sensitivity and specificity for the diagnosis of dVIN. Among p53, CK17, and SOX2, the highest sensitivity was obtained for SOX2 (86%), whereas, the highest specificity was obtained for p53 (83%) [supplement 4]. We therefore believe that CK17 and SOX2, when used in a panel along with p53, can complement the pathological assessment of dVIN. However, CK17 and SOX2, cannot be used to discriminate dVIN from HSIL. Moreover, for both CK17 and SOX2, increased expression was observed in a number of non-dysplastic lesions, whereas, a number of dVIN showed minimal expression. Therefore, for discriminating dVIN from non-dysplastic lesions, CK17 and SOX2 should be interpreted in the context of histology and clinical information, and should not be used as the sole diagnostic criterion. Further studies may help determine whether non-dysplastic lesions that show CK17 and SOX2 expression harbor molecular perturbations associated with early pre-neoplastic changes that precede histological manifestation.

In this study, we also investigated the molecular profile of a subset of dVIN and de-VIL, with a view to facilitate the identification of other potential diagnostic markers. In dVIN, *TP53* and *CDKN2A* mutations were detected most frequently, in line with previous reports [40,56-61]. Interestingly, *TP53* mutations were also detected in de-VIL, and dVIN that showed wild-type-expression on p53-IHC. Discordant results of *TP53* mutations and p53-IHC have been previously reported for dVIN [22]. Akbari et al. also detected *TP53* mutations of uncertain significance in some of the de-VIL in their cohort [24]. The higher frequency of *TP53* mutations in our cohort could be explained by the greater sequencing depth of our NGTS panel, which covers all exonic sites of *TP53*. In de-VILs, we could detect *PIK3CA* mutations, which have been reported to characterize these lesions [23,24]. However, these were not present in all de-VILs. Other interesting findings from the NGTS were – (i) frequent detection of *RNF43* mutations in both dVIN and de-VIL, which have been previously associated with endometrial carcinoma [62], and (ii) detection of mutations in *KEAP1*, *CDH1*, *PIK3R1*, and *POLE* exclusively in de-VIL. We also observed that a large number of the mutated genes in both dVIN and de-VIL could be mapped to the PI3K/AKT/mTOR pathway [63], indicating a potential involvement of this pathway in HPV-independent VSCC carcinogenesis. Nevertheless, to determine the clinical significance of these preliminary findings, further investigation in larger cohorts is necessary.

There are several limitations associated with the current study. As this is a retrospective, single-center study, a selection bias cannot be ruled out. Our findings need to be validated in prospective, multi-center cohorts. The IHC-markers were assessed by visual estimation, but computerized image analyses may offer higher accuracy. Furthermore, our NGTS panel comprised a limited number of amplicons, and the detections of mutations can be influenced by the platform used.

5. Conclusion

CK17 and SOX2 show significantly higher expression in dVIN (both p53-mutant and p53-wild-type) compared to non-dysplastic vulvar tissues. Particularly for lesions where dVIN is suspected on histology, and p53-expression is wild-type, diffuse, strong expression of CK17 and SOX2 can help confirm the diagnosis of dVIN. Therefore, we infer that CK17 and SOX2 can be useful adjuncts to p53 for the diagnosis of dVIN.

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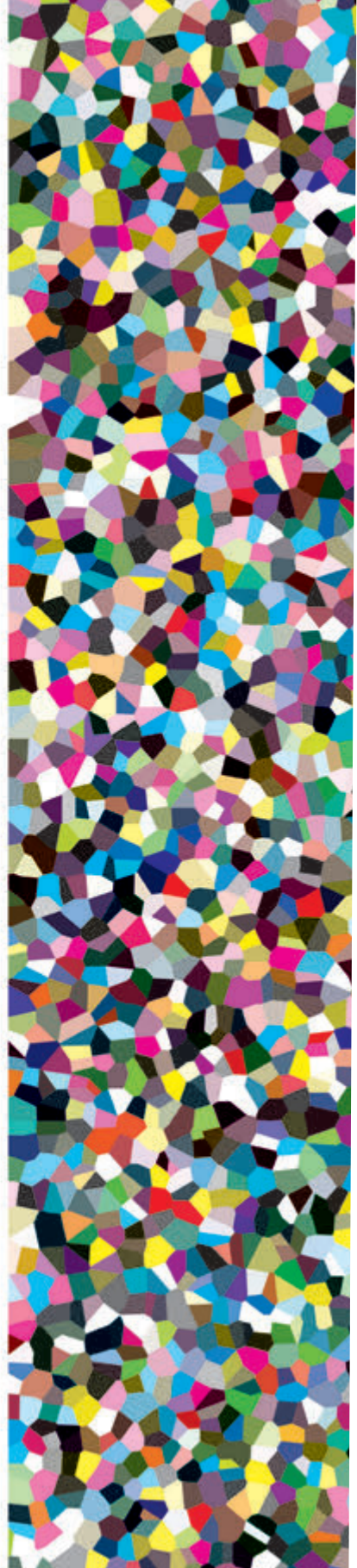
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7



CHAPTER 7

NUCLEAR FACTOR IB IS DOWNREGULATED IN VULVAR SQUAMOUS CELL CARCINOMA (VSCC) – UNRAVELLING DIFFERENTIALLY EXPRESSED GENES OF VSCC THROUGH ANALYSES OF GENE EXPRESSION DATASETS

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Abstract

Vulvar squamous cell carcinoma (VSCC) comprises two distinct etiopathological subtypes: i) Human papilloma virus (HPV)-related VSCC, which arises via the precursor high grade squamous intraepithelial lesion (HSIL); and ii) HPV-independent VSCC, which arises via precursor, differentiated vulvar intraepithelial neoplasia (dVIN), driven by *TP53* mutations. However, the mechanism of carcinogenesis of VSCC is poorly understood.

The current study aimed to gain insight into VSCC carcinogenesis by identifying differentially expressed genes (DEGs) for each VSCC subtype. The expression of certain DEGs was then further assessed by performing immunohistochemistry (IHC) on whole tissue sections of VSCC and its precursors. Statistical analysis of microarrays was performed on two independent gene expression datasets (GSE38228 and a study from Erasmus MC) on VSCC and normal vulva. DEGs were identified that were similarly (up/down) regulated with statistical significance in both datasets. For HPV-related VSCCs, this constituted 88 DEGs, and for HPV-independent VSCCs, this comprised 46 DEGs. IHC was performed on VSCC (n = 11), dVIN (n = 6), HSIL (n = 6) and normal vulvar tissue (n = 7) with i) signal transducer and activator of transcription 1 (STAT1; an upregulated DEGs); ii) nuclear factor IB (NFIB; a downregulated DEG); iii) p16 (to determine the HPV status of tissues); and iv) p53 (to confirm the histological diagnoses).

Strong and diffuse NFIB expression was observed in the basal and para-basal layers of normal vulvar tissue, whereas NFIB expression was minimal or completely negative in dVIN and in both subtypes of VSCC. In contrast, no discernable difference was observed in STAT1 expression among normal vulvar tissue, dVIN, HSIL or VSCC. By leveraging bioinformatics, the current study identified DEGs that can facilitate research into VSCC carcinogenesis. The results suggested that NFIB is down-regulated in VSCC and its relevance as a diagnostic/prognostic biomarker deserves further exploration.

1. Introduction

Vulvar squamous cell carcinoma (VSCC) constitutes 90% of all vulvar malignancies, and its incidence has risen over the past decades [1,2]. Approximately 25% of VSCCs arise in association with a human papillomavirus (HPV)-infection, via the precursor lesion, high grade squamous intraepithelial lesion (HSIL) [3]. The majority (75%) of VSCCs, however, is postulated to develop on the background of chronic dermatoses, via the precursor lesion, differentiated vulvar intraepithelial neoplasia (dVIN) [3].

The dual pathogenesis of VSCC has been recognized several years ago, however, molecular mechanisms of the carcinogenesis have not been well characterized [4]. This is largely because the genomic profiles of VSCC or its precursor lesions have been investigated in only a few studies so far [1,5-11].

These studies identified somatic mutations of *TP53* to be the pivotal oncogenic driver of HPV-independent VSCC, and also detected genomic alterations of *PIK3CA*, *HRAS*, or *FGFR3* in both subtypes of VSCC [7-9,11]. Nevertheless, limited sample sizes and dissimilar methodologies of these studies have prevented significant advancement of knowledge of VSCC carcinogenesis [4].

A better understanding of the molecular pathways involved in VSCC carcinogenesis can enable identification of biomarkers that may be used to improve the diagnosis, for prognostic stratification, or as targets for precision treatment. Currently, the mainstay of VSCC treatment is surgical excision, which is often associated with post-operative morbidities due to the anatomical complexity of the vulvar region. Discovering novel biomarkers for targeted treatment may help improve personalization of treatment for patients with VSCC.

A key method for discovering candidate biomarkers is through identifying genes that are differentially expressed in cancer tissue and normal tissue [12]. To this end, we analyzed datasets of gene expression microarray on VSCC and normal vulvar tissue, from two independent studies, using the latest bioinformatics tools. We further investigated the expression of some of the differentially expressed genes (DEGs) identified thereby, by performing immunohistochemistry (IHC) on VSCC, HSIL, dVIN, and normal vulvar tissue.

2. Materials and methods

2.1 Identification and analysis of datasets

A publicly available dataset (GSE38228) was identified and downloaded from gene expression omnibus (GEO) (13). This dataset consists of VSCCs (n = 14) and normal vulvar tissues (n = 5), for which gene expression microarray had been performed using the gene-chip platform Illumina HumanHT-12 V4.0. A 2nd dataset was obtained from a study previously conducted by researchers at our center. This dataset consists of VSCCs (n = 5), for which gene expression microarray was performed using the gene-chip platform Affymetrix HG U133 Plus 2.0. The datasets were imported into OmniViz (version 6.1.13.0, BioWisdomLtd.). Statistical analysis of microarrays (SAM) was performed to identify DEGs using the following cutoff values – a false discovery rate (FDR) of ≤ 0.01 and a fold change of 1.5. P-value < 0.05 was considered as statistically significant. Functional annotations of the SAM results were done using Ingenuity Pathway Analysis (IPA, Qiagen, Inc.).

Expression levels of p16 (*CDKN2A*), which is known to be overexpressed in HPV-related VSCC, were used to distinguish the samples as HPV-related or HPV-independent VSCC. For both subtypes of VSCC, DEGs that were upregulated or downregulated in both datasets with statistical significance were identified. The Database for Annotation, Visualization and Integrated Discovery (DAVID; version 6.8) was used to identify the most significantly enriched functional genes [14,15]. Gene ontology (GO) enrichment analyses were performed using the DAVID online tool to annotate biological process, cellular component, and molecular function of DEGs. Additional information on the DEGs was obtained from IPA, cBioPortal, and Gene Expression Profiling Interactive Analysis (GEPIA). Protein-protein interaction (PPI) networks of the DEGs were constructed using Search Tool for the Retrieval of Interacting Genes (STRING) [16-19].

2.2 Immunohistochemistry (IHC)

Formalin fixed paraffin embedded (FFPE)-tissues of VSCC, HSIL, dVIN, and normal vulva were retrieved from the archives of Department of Pathology, Erasmus MC. Histology of all tissues was reviewed by two pathologists (SDG and PCEG). Patient data were anonymized and patient materials were handled following the guidelines of World Medical Association Declaration of Helsinki.

For performing IHC, DEGs were selected – i) that were expressed in the cytoplasm or nucleus and ii) for which primary antibodies were commercially available. In addition, for all samples, IHC was performed with p16 to determine the HPV-status, and with p53 to confirm the histological diagnoses.

Sequential sections of 4 µm-thickness were prepared from the FFPE-tissues and automated IHC was performed using the Ventana Benchmark ULTRA (Ventana Medical Systems Inc.), following the manufacturer's instructions (Data S1 and Table S1).

The IHC markers were scored as follows:

For the IHC markers of DEGs, the percentage of cells showing staining, irrespective of the intensity of staining, was assessed manually. In addition, the intensity of staining (weak, moderate, and strong) and the distribution of staining within the epithelium was recorded.

p16-expression patterns were scored as block-type or non-block-type (patchy), following the guidelines of Lower Anogenital Squamous Terminology Standardization Project (LAST) [16]. Block-type p16-expression, i.e. diffuse, continuous, moderate-to-intense nuclear and/or cytoplasmic staining in $\geq 1/3$ rd of the epithelial thickness is considered to be a reliable surrogate marker of high-risk HPV-infection [20].

p53-expression patterns were scored as p53-mutant or p53-wild-type following descriptions in recent literature [10,21]. p53-mutant patterns have been reported to accurately reflect the presence of *TP53* mutations [10]. p53-mutant patterns include basal to parabasal/diffuse overexpression, basal overexpression, or aberrant negative/null-pattern. p53-wild-type patterns include scattered heterogeneous basal and/or para-basal expression, and scattered mid-epithelial expression with basal sparing. The latter p53-wild-type pattern is associated with HPV-related lesions [10,22].

2.3 Ethics statement

This study was conducted in accordance with the guidelines of the Dutch Federation of Biomedical Scientific Societies (www.federa.org/codes-conduct), which state that no separate ethical approval is required for the use of anonymized residual tissue procured during regular treatment.

3. Results

3.1 Dataset analyses

From GSE38228, 3 samples were identified as HPV-related VSCC and 3 samples were identified as HPV-independent VSCC. A total of 342 genes (244 upregulated and 98 downregulated) were found to be differentially expressed with statistical significance only in HPV-related VSCC. A total of 382 genes (203 upregulated and 179 downregulated) were found to be differentially expressed with statistical significance only in HPV-independent VSCC. From the 2nd dataset, 3 samples were identified as HPV-related VSCC and 2 samples as HPV-independent VSCC. A total of 7005 genes were differentially expressed with statistical significance in HPV-related VSCC, and 4283 genes were differentially expressed with statistical significance in HPV-independent VSCC. Combining both datasets, for HPV-related VSCC, 88 DEGs were identified that were similarly regulated with statistical significance. This comprised 69 upregulated and 19 downregulated DEGs; signal transducer and activator of transcription 1 (STAT1) was one of the upregulated DEGs. For HPV-independent VSCC, 46 DEGs were identified that were similarly regulated with statistical significance. This comprised 16 upregulated and 30 downregulated DEGs; nuclear factor IB (NFIB) was one of the downregulated DEGs. The PPI networks of these DEGs are visualized in Figures 1 and 2, and the DEGs along with their subcellular locations, functions, and related canonical pathways are listed in Tables S2 and S3.

The DEGs identified for HPV-related VSCC mainly participate in response to stimulus and regulation of cellular and biological processes [Table 1]. As for the molecular function, these DEGs are mainly involved in binding with ions or signaling receptors [Table 2]. The cellular component of these DEGs include cytoplasm and extracellular region. The DEGs identified for HPV-independent VSCC mainly participate in regulation of cellular and metabolic processes [Table 3]. As for the molecular function, these DEGs are mainly involved in protein and ion binding [Table 4]. The cellular component of these DEGs include membrane-bound organelles and the cytoplasm.

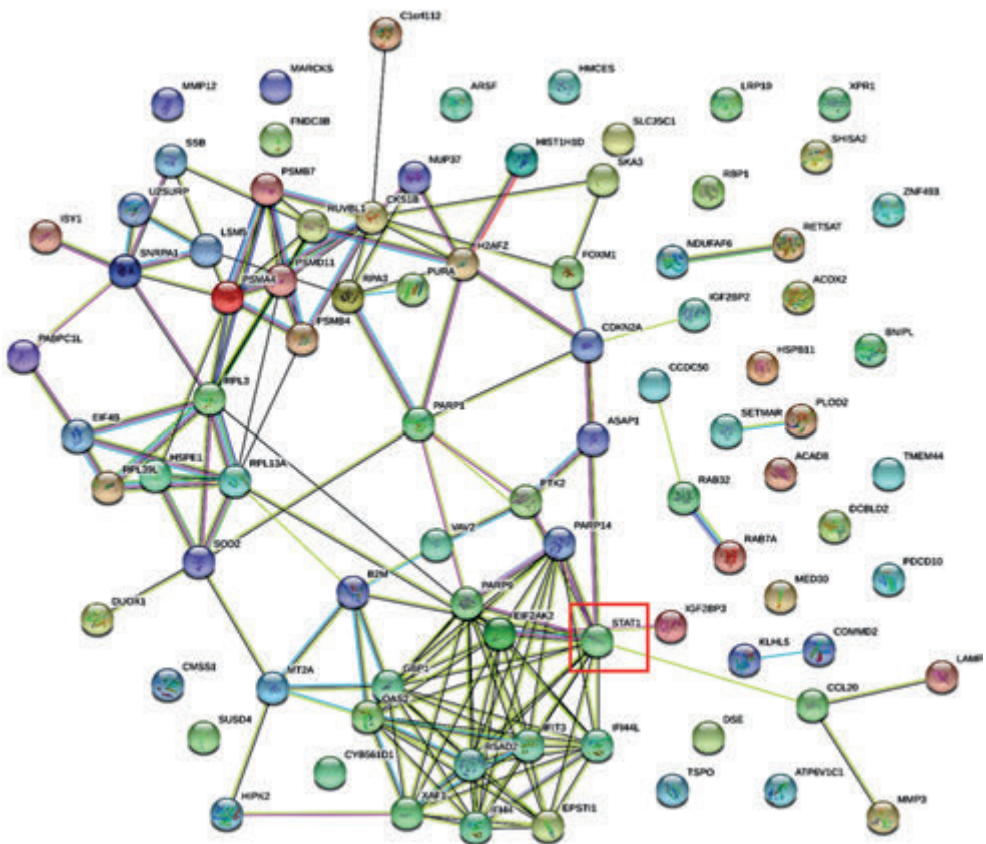


Figure 1. Protein-protein interaction network of the 88 differentially expressed genes in HPV-related VSCC (constructed with STRING). Signal transducer and activator of transcription 1 is indicated with a red box. The network nodes represent the proteins produced by a single, protein-coding gene locus. Colored nodes represent query proteins and first shells of interactions. White nodes represent second shell of interactions. Empty nodes represent proteins of an unknown 3D structure. Filled nodes represent proteins of which some 3D structures are known or predicted. The edges are coded as follows: Light blue, known interaction curated from databases; pink, known interaction determined through experiments; green, predicted interaction in the gene neighborhood; red, gene fusions; dark blue, gene co-occurrence; lime green, text mining; black, co-expression; indigo, protein homology

Table 1: GO enrichment analysis of the differentially expressed genes for HPV-related VSCC

Term	Description	Gene count	p-value
<i>Biological processes</i>			
GO:0050896	response to stimulus	35	2.89E-10
GO:0050794	regulation of cellular process	34	2.92E-05
GO:0050789	regulation of biological process	34	1.19E-04
GO:0032501	multicellular organismal process	33	6.85E-11
GO:0007275	multicellular organism development	26	1.39E-08
GO:0048856	anatomical structure development	26	7.60E-08
GO:0042221	response to chemical	25	8.56E-09
GO:0007154	cell communication	24	1.09E-05
GO:0006950	response to stress	23	8.86E-09
GO:0007165	signal transduction	23	6.01E-06
<i>Molecular functions</i>			
GO:0043167	ion binding	29	2.59E-08
GO:0005102	signaling receptor binding	21	2.35E-13
GO:0097367	carbohydrate derivative binding	19	4.81E-09
GO:0043169	integrin binding	19	1.11E-04
GO:0098772	molecular function regulator	18	6.66E-05
GO:0008201	heparin binding	15	1.60E-21
GO:1901681	sulfur compound binding	15	9.93E-19
GO:0030545	receptor regulator activity	12	2.24E-10
GO:0005198	structural molecule activity	9	5.41E-06
GO:0005509	growth factor binding	8	5.60E-05
<i>Cellular component</i>			
GO:0005737	cytoplasm	33	4.88E-04
GO:0005576	extracellular region	30	1.54E-13
GO:0012505	endomembrane system	24	1.73E-07
GO:0031982	vesicle	20	5.59E-06
GO:0043230	extracellular organelle	14	1.66E-05
GO:0031410	cytoplasmic vesicle	14	8.38E-05
GO:0097708	intracellular vesicle	14	8.57E-05
GO:0062023	collagen-containing extracellular matrix	13	6.10E-13
GO:0070062	extracellular exosome	13	7.18E-05
GO:1903561	extracellular vesicle	13	7.96E-05

Table 2: Functional annotation analysis of the differentially expressed genes for HPV-related VSCC

Functional annotation clusters	Gene count	p-value
Immunity	8	5E-06
Antiviral defense	7	9E-09
Defense response to virus	7	2E-07
Host-virus interaction	6	2E-05
Protease	6	8E-05
Type I interferon signaling	5	3E-07
Innate immunity	5	2E-05
Perinuclear region of cytoplasm	5	3E-05
Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	5	2E-07
Tumor necrosis factor-mediated signaling	5	3E-06

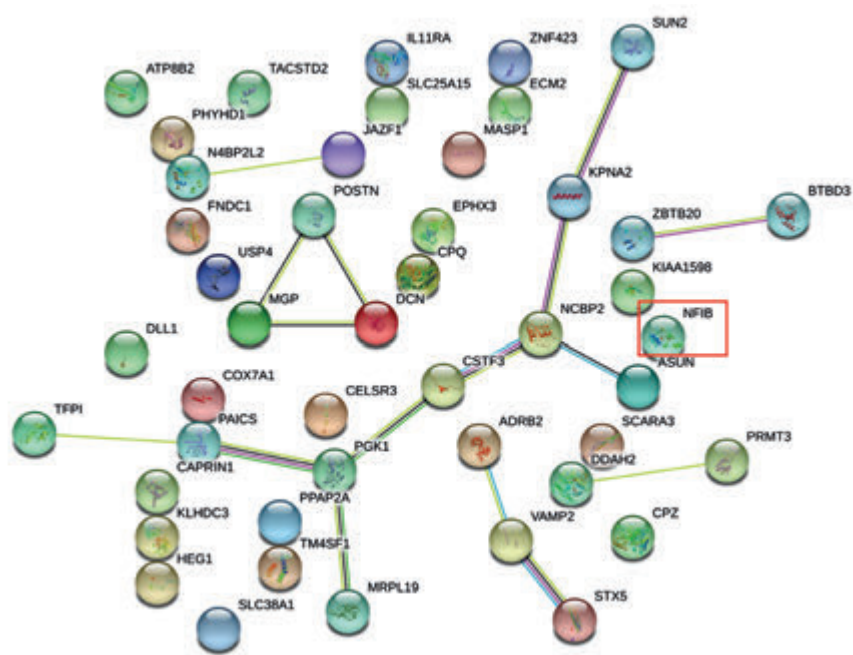


Figure 2. Protein-protein interaction network of the 46 differentially expressed genes in HPV-independent VSCC (constructed with STRING). Nuclear factor IB is indicated with a red box. Network nodes represent all the proteins produced by a single, protein-coding gene locus. Colored nodes represent query proteins and first shells of interactions. White nodes represent second shell of interactions. Empty nodes represent proteins of an unknown 3D structure. Filled nodes represent proteins of which some 3D structures are known or predicted. The edges are coded as follows: Light blue, known interaction curated from databases; pink, known interaction determined through experiments; green, predicted interaction in the gene neighborhood; red, gene fusions; dark blue, gene co-occurrence; lime green, text mining; black, co-expression; indigo, protein homology.

3.2 Immunohistochemistry

Primary antibodies for performing IHC were commercially available for i) STAT1, one of the upregulated DEGs, and ii) NFIB, one of the downregulated DEGs. IHC was performed on 11 VSCCs, 6 dVINs, 6 HSILs, and 7 normal vulva tissues; these were from women with a median age of 72.5 years (range: 26 – 90 years). Immunohistochemical expression of p53, p16, NFIB, and STAT1 are presented in Tables 5 and 6. For NFIB and STAT1, the IHC patterns observed in the tissues are described below, and the distribution of expression is depicted in Figure S1.

STAT1: Normal vulvar tissue (n = 7): Five showed diffuse, cytoplasmic STAT1-expression of moderate-to-strong intensity, across full epithelial thickness; two showed focal STAT1 expression of moderate-to-strong intensity.

dVIN (n = 6), HSIL (n = 6), HPV-related VSCC (n = 5), HPV-independent VSCC (n = 6): All showed diffuse, cytoplasmic STAT1-expression of moderate-to-strong intensity, across full epithelial thickness.

NFIB: Normal vulvar tissue (n = 7): All showed strong, diffuse, nuclear NFIB-expression, predominantly along the basal layers, which occasionally extended to the parabasal layers.

HSIL (n = 6): All showed strong nuclear NFIB-expression along the basal layers and occasionally in the parabasal layers. Staining in the basal layer was discontinuous, and expression in the parabasal layers was primarily seen only at the tips of rete ridges.

HPV-related VSCC (n = 5): Two were completely negative, and 2 were predominantly negative, showing only focal, weak, nuclear NFIB-expression along the periphery of the tumor cell nests. One VSCC showed NFIB-expression of moderate intensity along the periphery of the tumor cell nests.

dVIN (n = 6): One dVIN was completely negative and 5 showed only focal, weak, nuclear NFIB-expression.

HPV-independent VSCC (n = 6): One was completely negative, and 5 were predominantly negative, showing only focal, weak, nuclear NFIB-expression along the periphery of the tumor cell nests.

Immunohistochemical expressions of p53, p16, STAT1, and NFIB in normal vulvar tissue, HSIL, dVIN, and VSCC (both subtypes) are demonstrated in Figures 3 – 7.

Table 3: GO enrichment and functional annotation analyses of DEGs for HPV-independent VSCC

Term	Description	Gene count	p-value
<i>Biological processes</i>			
GO:0050794	regulation of cellular process	33	5.27E-12
GO:0008152	metabolic process	31	1.02E-11
GO:0016043	cellular component organization	25	7.37E-09
GO:0032502	developmental process	22	3.69E-11
GO:0032501	multicellular organismal process	19	4.55E-11
GO:0065008	regulation of biological quality	19	6.93E-12
GO:0007275	multicellular organism development	19	7.01E-08
GO:0007154	cell communication	18	9.79E-06
GO:0030154	cell differentiation	18	1.04E-06
GO:0042221	transport	17	8.87E-10
<i>Molecular functions</i>			
GO:0005515	protein binding	38	5.43E-07
GO:0043167	ion binding	20	7.70E-10
GO:0003824	catalytic activity	13	1.00E-11
GO:0097159	organic cyclic compound binding	12	7.47E-09
GO:1901363	heterocyclic compound binding	11	6.24E-09
GO:0003676	nucleic acid binding	8	8.53E-05
GO:0016787	hydrolase activity	7	5.01E-12
GO:0043168	anion binding	7	8.30E-10
GO:0098772	molecular function regulator	7	7.05E-06
GO:0097367	carbohydrate derivative binding	6	6.35E-05
<i>Cellular component</i>			
GO:0043227	membrane-bounded organelle	36	2.20E-09
GO:0005737	cytoplasm	31	1.82E-09
GO:0016020	cell membrane	26	2.41E-11
GO:0005576	extracellular region	20	7.74E-05
GO:0031224	intrinsic component of membrane	20	3.26E-06
GO:0005634	nucleus	18	7.60E-11
GO:0031982	vesicle	14	5.76E-05
GO:0071944	cell periphery	14	7.46E-07
GO:0043233	organelle lumen	14	8.72E-12
GO:0012505	endomembrane system	13	3.75E-10

Table 4: Functional annotation analysis of differentially expressed genes for HPV-independent VSCC

Functional annotation clusters	Gene count	p-value
Developmental protein	21	5E-07
Calcium ion binding	16	2E-05
EGF-like domain	12	1E-04
EGF-like calcium-binding, conserved site	11	7E-06
Cell-cell adhesion	7	2E-04
Integral component of membrane	6	5E-05
Cell differentiation	5	1E-04
Acetylation	4	1E-05
Extracellular matrix organization	3	2E-05
Transmembrane helix	3	6E-04

Table 5: Immunohistochemical expression patterns of p53 and p16

Marker and expression pattern	Diagnosis				
	Normal vulvar tissue (n = 7)	HSIL (n = 6)	HPV-related VSCC (n = 5)	dVIN (n = 6)	HPV-independent VSCC (n = 6)
p53-mut					
Parabasal / Diffuse	0 (0)	0 (0)	1 (20)	5 (83)	3 (50)
Basal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Absent / Null	0 (0)	0 (0)	0 (0)	0 (0)	2 (33)
p53-wt					
Wild-type scattered	7 (100)	0 (0)	0 (0)	1 (17)	1 (17)
Wild-type mid-epithelial	0 (0)	6 (100)	4 (80)	0 (0)	0 (0)
p16					
Block-type	0 (0)	6 (100)	5 (100)	0 (0)	0 (0)
Non-block-type / patchy	0 (0)	0 (0)	0 (0)	1 (17)	0 (0)
No expression	7 (100)	0 (0)	0 (0)	5 (83)	6 (100)

Data are presented as n (%). HSIL, high grade squamous intraepithelial lesions; HPV, human papilloma virus; VSCC, vulvar squamous cell carcinoma; dVIN, differentiated vulvar intraepithelial neoplasias; mut, mutant; wt, wild-type.

Table 6: Immunohistochemical expression of NFIB and STAT1

Marker and expression \ pattern	Diagnosis				
	Normal vulvar tissue (n = 7)	HSIL (n = 6)	HPV-related VSCC (n = 5)	dVIN (n = 6)	HPV-independent VSCC (n = 6)
NFIB	18 (10.3-23.1)	12.5 (9.1-17.6)	5 (1.1-9.2)	6 (4.3-10.1)	2.5 (0.9-11.3)
STAT1	65 (50.8-87.5)	67.5 (38.8-81.2)	80 (68.5-91.5)	85 (69.1-92.5)	90 (53.1-95.6)

Data are presented as the mean with 95% confidence interval. NFIB, nuclear factor IB; STAT1, signal transducer and activator of transcription 1; HSIL, high grade squamous intraepithelial lesions; HPV, human papilloma virus; VSCC, vulvar squamous cell carcinoma; dVIN, differentiated vulvar intraepithelial neoplasias.

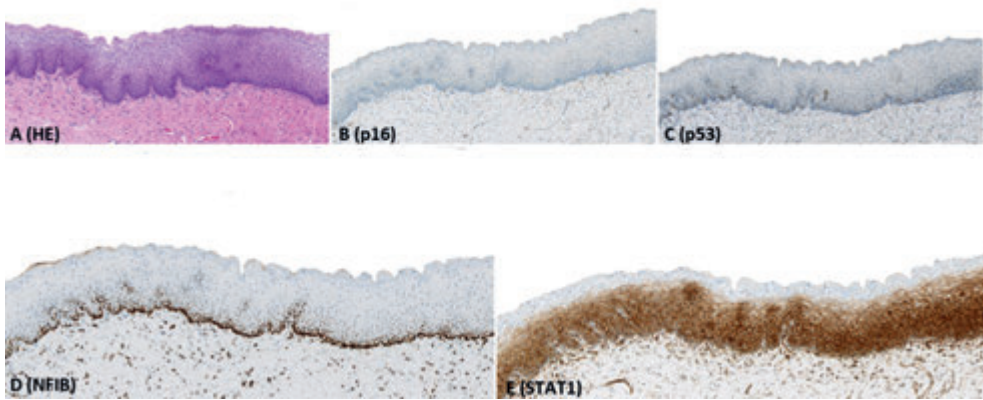


Figure 3: Normal vulvar tissue histology and IHC. A. Histological appearance (hematoxylin and eosin stain); B. p16-IHC was negative; C. p53-IHC revealed wild-type expression; D. NFIB-IHC exhibited strong, diffuse, nuclear expression, predominantly along the basal layers and occasionally in the parabasal layers; E. STAT1-IHC demonstrated diffuse, cytoplasmic expression of moderate-to-strong intensity, across full epithelial thickness (A-C, magnification, x100; D and E, magnification, x200). IHC, immunohistochemistry.

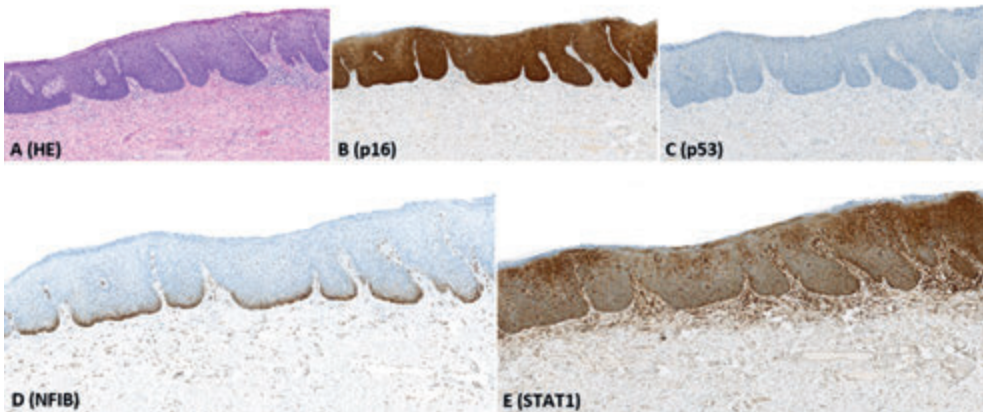


Figure 4: HSIL – histology and IHC A. Histological appearance (HE-stain); B. p16-IHC shows block-type expression; C. p53-IHC shows wild-type expression; D. NFIB-IHC shows strong nuclear expression along the basal layers and occasionally in the parabasal layers. Staining in the basal layer is discontinuous, and expression in the parabasal layers is mainly limited to the tips of rete ridges; E. STAT1-IHC shows diffuse, cytoplasmic expression of moderate-to-strong intensity, across full epithelial thickness (A-C: original magnification 100X; D-E: original magnification 200X).

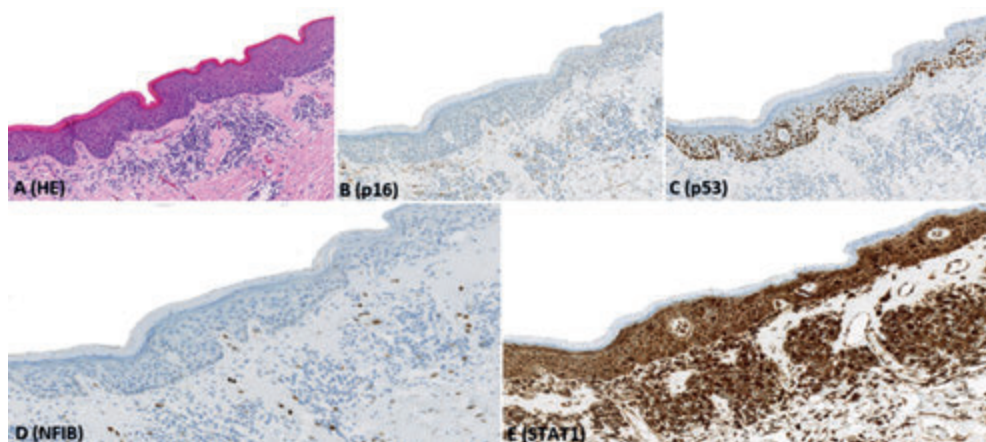


Figure 5: dVIN – histology and IHC A. Histological appearance (HE-stain); B. p16-IHC is completely negative; C. p53-IHC shows mutation-pattern; D. NFIB-IHC is completely negative; E. STAT1-IHC shows diffuse, cytoplasmic expression of moderate-to-strong intensity, across full epithelial thickness (A-C: original magnification 100X; D-E: original magnification 200X).

4. Discussion

In this study, we utilized bioinformatics tools to gain insight into VSCC carcinogenesis, and to identify potential biomarkers that may have diagnostic, prognostic, or therapeutic applications. For both subtypes of VSCC (i.e., HPV-related and HPV-independent) we identified a set of DEGs that appeared to be similarly regulated (up or down) in two independent gene expression microarray datasets. We found that the majority of DEGs that were identified for HPV-related VSCC are involved in the immune response, whereas those identified for HPV-independent VSCC were involved in second messenger signaling-this provides support for the dual pathogenesis of VSCC.

We studied the expression of two of the DEGs, i.e. NFIB and STAT1, that were found to be similarly regulated in both datasets, in whole tissue sections of VSCCs, dVINS, HSILs, and normal vulvar tissues, by performing IHC. NFIB was identified to be downregulated in HPV-independent VSCC, and STAT1 was identified to be upregulated in HPV-related VSCC. Neither of these markers has been previously studied for VSCC or its precursor lesions.

NFIB showed strong, nuclear expression in the basal and parabasal epithelial layers in normal vulvar tissue, whereas, in dVIN and both subtypes of VSCC, NFIB was either completely negative or minimally expressed. NFIB expression was

also reduced in HSIL in comparison with normal vulvar tissue, but to a lesser extent than that in dVIN and VSCC.

NFIB is a transcription factor which has tumor suppressive, as well as, oncogenic potential [23]. In cervical SCC and head-and-neck SCC (HNSCC), NFIB-expression has been observed to be lower than in normal tissues from the corresponding sites [23]. Furthermore, lower levels of NFIB-expression have been reported to correlate significantly with worse prognosis for both of these malignancies [23]. Interestingly, NFIB is a key regulator of the aryl hydrocarbon pathway, which we previously identified to be involved in HPV-independent VSCC [2]. In addition, high-confidence proximity interactions have been reported between NFIB and SOX2 [24]; SOX2 is a cancer-stemness related transcription factor that is overexpressed in dVIN and VSCC [25]. In view of these observations, we believe that the role of NFIB in VSCC and its potential as a therapeutic target deserve further investigation. In addition to SCCs, genomic alterations of NFIB have been detected in several other malignancies, as shown in Figure S2.

Unlike NFIB, no discernable difference was observed in immunohistochemical expression of STAT1 between normal vulvar tissue, dVIN, HSIL, or VSCC (both subtypes). For all tissue types, diffuse, cytoplasmic STAT1-expression of moderate-to-strong intensity was noted across full epithelial thickness. STAT1 is a component of the Janus kinase (JAK)-STAT signaling pathway, and can act as an antimicrobial mediator, a tumor suppressor, or a promotor of tumor progression [26]. Aberrant expression of STAT1 in HPV-related lesions is considered to reflect activation of the JAK-STAT pathway as a consequence of the inflammatory response induced by HPV [26].

Our results regarding IHC-expression of STAT1 were in contrast to those of a recent study, which reported a higher STAT1-expression in cervical intraepithelial neoplasia (CIN) than in normal cervical epithelium, and deduced an association of increased STAT1-expression with malignant progression of CIN [26]. Since STAT-1 expression is regulated by a complex network of interferons, we speculate that the difference in expression between vulvar and cervical tissue could be ascribed to the dissimilar microenvironments of these anatomical sites. Similarly to NFIB, genomic alterations of STAT1 have been detected in several malignancies, as shown in Figure S3.

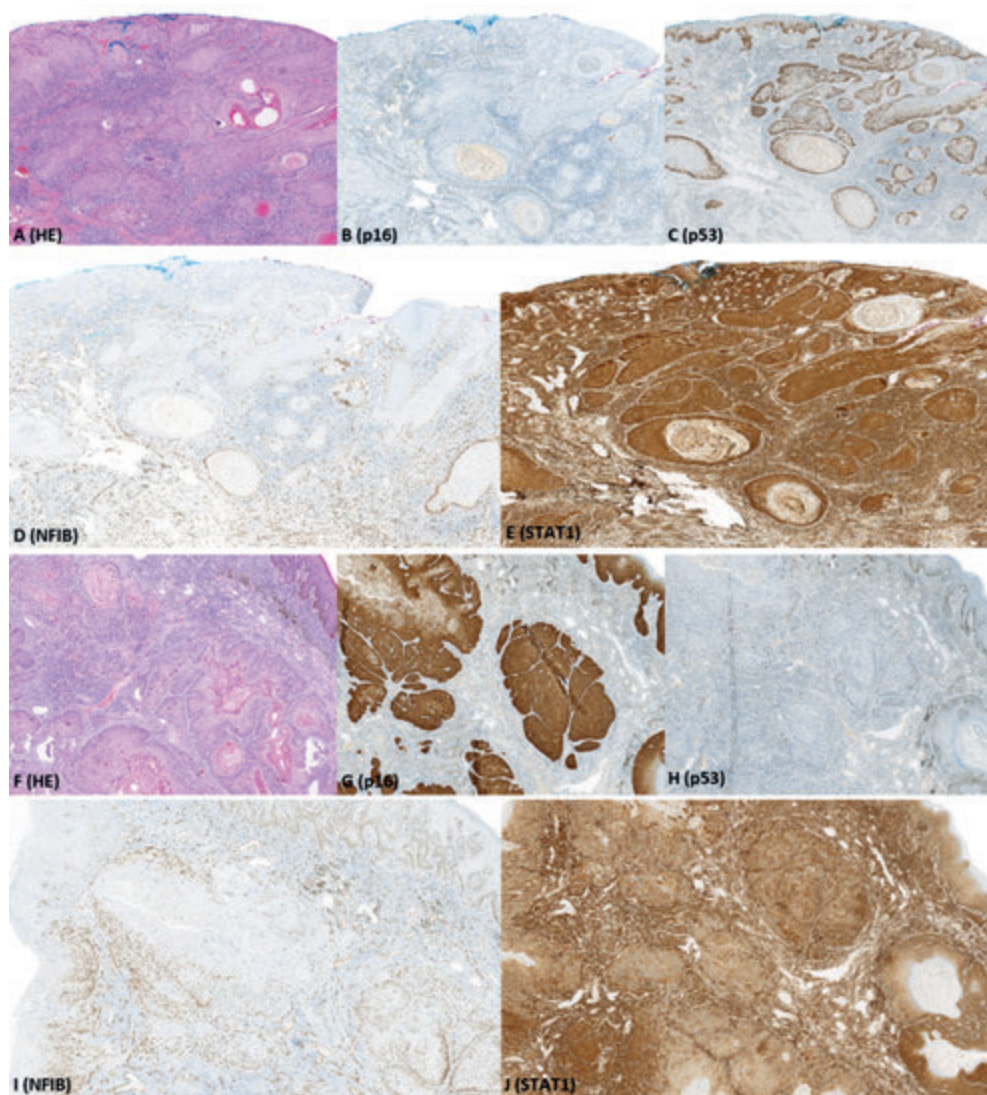


Figure 6: VSCC – histology and IHC A. Histological appearance of HPV-related VSCC (HE-stain); B. p16-IHC shows block-type expression; C. p53-IHC shows wild-type expression; D. NFIB-IHC is negative in some tumor nests, and shows focal, weak, nuclear expression along the periphery of some of the tumor nests; E. STAT1-IHC shows diffuse, cytoplasmic expression of moderate-to-strong intensity, across full epithelial thickness; F. Histological appearance of HPV-independent VSCC (HE-stain); G. p16-IHC is completely negative; H. p53-IHC shows mutation-pattern; I. NFIB-IHC is negative in some tumor nests, and shows focal, weak, nuclear expression along the periphery of some of the tumor nests; J. STAT1-IHC shows diffuse, cytoplasmic expression of moderate-to-strong intensity, across full epithelial thickness (A-C, F-H: original magnification 100X; D-E, I-J: original magnification 200X).

5. Conclusion

This study was an attempt to leverage bioinformatics to identify DEGs in VSCC. We identified NFIB as a downregulated gene in VSCC, and observed that its immunohistochemical expression was reduced in both subtypes of VSCC. Hence, we believe that the relevance of NFIB as a diagnostic/prognostic biomarker deserves further exploration. However, an apparent limitation of this study is that the DEGs were identified from datasets consisting of small sample sizes, and IHC was also performed on a limited set of tissues. Further experiments are needed to confirm the function of these DEGs in VSCC and to validate their immunohistochemical expression in vulvar tissues.

Nevertheless, we hope that our results will instigate further research into VSCC carcinogenesis and pave the path for unravelling novel biomarkers of VSCC and its precursor lesions.

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CHAPTER 8

EXPLORING DIFFERENTIALLY METHYLATED GENES IN VULVAR SQUAMOUS CELL CARCINOMA

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Abstract

DNA-methylation is the most widely studied mechanism of epigenetic modification, which can influence gene-expression without alterations in DNA sequences. Aberrations in DNA-methylation are known to play a role in carcinogenesis, and methylation profiling has enabled the identification of biomarkers of potential clinical interest for several cancers. For vulvar squamous cell carcinoma (VSCC) however, methylation profiling remains an under-studied area.

We sought to identify differentially methylated genes (DMGs) in VSCC, by performing Infinium MethylationEPIC BeadChip (Illumina) array sequencing, on a set of primary VSCC (n = 18), and normal vulvar tissue from women with no history of vulvar (pre)malignancies (n = 6). Using false-discovery rate of 0.05 and beta-difference ($\Delta\beta$) of ± 0.5 as cut-offs, 199 DMGs (195 hyper-methylated, 4 hypo-methylated) were identified for VSCC. The majority of the hyper-methylated genes were found to be involved in transcription regulator activity, indicating that disruption of this process plays a role in VSCC development. The majority of VSCCs harbored amplifications of chromosomes 3, 8, and 9.

We identified a set of DMGs in this exploratory, hypothesis-generating study, which we hope will facilitate epigenetic profiling of VSCCs. Prognostic relevance of these DMGs deserves further exploration in larger cohorts of VSCC and its precursor lesions.

1. Introduction

Vulvar squamous cell carcinoma (VSCC) is considered to be a rare cancer, however, an increase in the incidence and a decrease in the median age of onset has been witnessed over the past decades [1-4]. VSCC comprises two main etiological subtypes – human papillomavirus (HPV)-associated and HPV-independent [5,6]. These subtypes are also known to differ in their epidemiological, clinical, pathological, and molecular characteristics. For example, HPV-associated VSCC, the less prevalent subtype, affects women of 50 – 60 years of age, and is associated with a favorable prognosis [7,8]. In contrast, the more prevalent HPV-independent VSCC usually develops on the background of chronic dermatoses in women of > 70 years of age, and is associated with high rates of recurrence [2,5-7,9]. On a molecular level, somatic mutations of *TP53* have been frequently detected in HPV-independent VSCCs, and have been implicated as the ‘oncogenic driver’ for this subtype [7,10-12]. However, recent studies have discovered molecular heterogeneity among HPV-independent VSCCs, as some of these tumors can be *TP53* wild-type, and harbor *HRAS* or *NOTCH1* mutations instead [7,10].

Although our understanding of VSCC carcinogenesis has progressed, treatment options for VSCC patients have not significantly evolved over the years [13,14]. The mainstay of VSCC treatment remains surgery with tumor-free resection margins, confirmed on microscopy [13-15]. Unfortunately, surgical interventions in the vulva may injure adjacent vital structures such as the urethra or the anus, resulting in post-operative morbidity and a reduced quality of life [16]. Effective therapeutic alternatives that may help avoid such adverse consequences are therefore an urgent unmet need.

For the identification of biomarkers of potential diagnostic, prognostic, predictive, or therapeutic interest, comprehensive molecular characterization of cancer, by comparing molecular profiles of cancer and normal tissue from the same anatomical site is essential [17]. In recent years, a number of studies have investigated genomic changes, e.g. somatic mutations and copy number variations in VSCC, and its precursor lesion, vulvar intraepithelial neoplasia (VIN) [7,10-12,18]. However, the epigenomic changes in VSCC remain relatively underexplored.

Epigenetic modifications, or changes in gene-expression without alterations in DNA sequences, can play a crucial role in carcinogenesis. Of the several

mechanisms of epigenetic modification, DNA-methylation, which involves binding of a methyl (-CH₃) group to the cytosine residues of the promoter cytosine-phosphate-guanine (CpG) dinucleotides, has been most widely studied. Aberrations of CpG island methylation have been implicated in the development of several gynecological and non-gynecological cancers [19-22].

In this exploratory study, we aimed to identify methylation-based biomarkers of potential clinical relevance by investigating the genes that are differentially methylated in VSCC compared to normal vulvar tissue. To this end, we performed genome-wide methylation sequencing on a set of VSCC, and normal vulvar tissues from women with no history of vulvar (pre)malignancies.

2. Materials and methods

2.1 Ethical clearance

This study follows the guidelines of the Dutch Federation of Biomedical Scientific Societies (www.federa.org/codes-conduct), which state that no separate ethical approval is required for the use of anonymized residual tissue procured during regular treatment. Approval for this study was also obtained from the Institutional Review Board of Erasmus MC (MEC-2020-0731).

2.2 Tissue procurement

Cases of primary VSCC treated with curative intent between 2016 and 2018 were identified from the electronic records of the Department of Pathology, Erasmus MC. Normal vulvar tissues were procured from women without any history of vulvar (pre)malignancies, who underwent surgery for benign vulvar pathologies. Hematoxylin-eosin (HE) stained glass-slides and formalin-fixed paraffin embedded (FFPE) tissues were retrieved from the archives.

2.3 Tissue processing

Twelve sections of 4µm thickness were prepared from the FFPE-tissues. The first and the last sections were stained with HE to confirm the diagnoses and / or the presence of the lesions. Eight of the remaining slides were stained with hematoxylin to extract tissue for DNA-isolation, and 2 were used for immunohistochemical staining.

2.4 Immunohistochemistry

Immunohistochemistry (IHC) was performed with p16 (CintecR®) and p53 (clone: Bp53-11) on an automated, validated staining system (Ventana Benchmark ULTRA, Ventana Medical Systems, Tucson, AZ, USA). Expression patterns of p16 and p53 were used to categorize the VSCCs as HPV-associated or HPV-independent. p16 is considered to be a reliable surrogate marker of high-risk HPV-infection, and aberrant patterns of p53-expression have been reported to reflect underlying *TP53* mutations [23-25].

p16 and p53 were scored following published literature [23-26], as described below.

p16: block-type expression = continuous, strong, nuclear and / or cytoplasmic expression involving $\geq 1/3$ rd of epithelial thickness; non-block type expression = patchy expression in clusters of cells; no expression = complete lack of expression.

p53: mutant patterns = diffuse (basal to parabasal) overexpression / basal overexpression / null-pattern / cytoplasmic expression; wild-type pattern (scattered) = scattered, heterogeneous, basal or parabasal expression; wild-type pattern (mid-epithelial) = heterogeneous, mid-epithelial expression with sparing of basal cells and / or lower parabasal cells.

2.5 DNA-isolation

DNA-isolation was performed on the Maxwell RSC DNA FFPE kit® (Promega Corporation, Madison, WI, US) following manufacturer's instructions. In brief, the regions of interest having a minimum lesional cell percentage of 60 were selected on the HE-stained slides. The corresponding areas were microdissected manually from the hematoxylin stained slides using sterile scalpel blades, and placed in sample tubes. Next, 300µl of mineral oil was added to the sample tubes, and heated at 80 °C for 2 minutes, followed by the addition of 250µl of master mix comprising lysis buffer, proteinase K, and blue dye. The tubes were then centrifuged at 10,000g for 20 seconds, heated at 56 °C for 30 minutes and incubated at 80 °C for 4 hrs. The samples were next incubated with RNaseA for 5 minutes at room temperature. The concentration of the extracted DNA was measured with the Qubit 2.0 fluorometer (Thermo FisherScientific, Waltham, MA, USA).

2.6 Methylation assay

Bisulfite-conversion and measurement of DNA-methylation was performed at the Human Genomics Facility (HUGE-F) of Erasmus MC. Bisulfite-conversion was performed on 250ng of genomic DNA extracted from the FFPE samples, using the EZ-96 DNA Methylation deep-well Kit (Zymo Research, Irvine, CA, USA). The samples were plated in a randomized order. Next, restoration was performed on the samples with the Infinium HD FFPE DNA Restore Kit (Illumina Inc., San Diego, CA, USA). Finally, the restored samples were hybridized on the Infinium MethylationEPIC BeadChip (Illumina Inc., San Diego, CA). DNA methylation was measured with the Infinium MethylationEPIC BeadChip (Illumina Inc., San Diego, CA), following manufacturer's protocol. The Illumina Infinium Methylation EPIC BeadChip Kit (Illumina, San Diego, CA, USA) is a high-throughput array that allows quantification of more than 850,000 methylation sites across the human genome at single nucleotide resolution.

2.7 Statistical analysis

For inclusion in the analysis, the following criteria were used – (i) samples that passed technical quality checks, such as, extension, hybridization, and bisulfite conversion according to the criteria set by Illumina, (ii) samples having a call rate of > 90%, and (iii) probes having a detection p-value > 0.05 or bead number > 3 for at least 10% of the samples. Beta (β)-values were used to measure the methylation probe intensity and the overall intensity. Differences in the β -values, or, $\Delta\beta$ -values were used to identify the differentially methylated probes; $\Delta\beta$ can assume any value between 0 and ± 1 . Methylation data were analyzed using PARTEK Inc 2020 (Version 10.0) Functional normalization of the data was performed. Principal component analysis (PCA) scatter plots were constructed for quality check. Analysis of variance (ANOVA) was performed to test the statistical significance of the differential methylation; two-sided p-value < 0.05 was considered significant. Unsupervised hierarchical clustering was performed to identify the differentially methylated probes. For functional annotation of the differentially methylated genes, (DMGs), DAVID [27,28] and Ingenuity Pathway Analysis (QIAGEN Inc.) were used. In addition, to visualize the ranges of β -values for specific DMGs, box-plots were constructed.

3. Results

3.1 Clinico-pathological information

Eighteen VSCC and 6 normal vulvar tissues were included in this study; clinico-pathological characteristics are presented in Table 1. Of the VSCCs, 3 (17%) showed block-type p16-expression, and were categorized as HPV-associated, while, 15 (83%) showed non-block-type or no p16-expression, and were categorized as HPV-independent. Of the HPV-independent VSCCs, 13 (87%) showed mutant patterns and 2 (13%) showed wild-type pattern of p53-expression [Table 1].

Table 1: Clinico-pathological characteristics

	Diagnoses		
	HPV-independent VSCC (n = 15)	HPV-associated VSCC (n = 3)	Normal vulvar tissue (n = 6)
Median Age (Range) in years	76 (52 – 90)	73 (65 – 81)	75 (52 – 90)
Location			
Vulvar skin with adnexa	8 (53)	1 (33)	1 (17)
Vulvar skin without adnexa	7 (47)	2 (67)	5 (83)
p16-expression			
Block-type	0 (0)	3 (100)	0 (0)
Non-block-type	8 (53)	0 (0)	1 (17)
No expression	7 (47)	0 (0)	5 (83)
p53-expression			
Diffuse overexpression	9 (60)	1 (33)	0 (0)
Basal overexpression	1 (7)	0 (0)	0 (0)
Null pattern	3 (20)	0 (0)	0 (0)
Wild-type expression (scattered)	2 (13)	0 (0)	6 (100)
Wild-type expression (mid-epithelial)	0 (0)	2 (67)	0 (0)

3.2 Differentially methylated genes

Of the 850,000 methylation probes of Illumina Infinium MethylationEPIC array, 66,069 probes interrogate CpG sites of all known genes as categorized by Illumina. Of these, using the cut-off of false discovery rate of 0.05 and $\Delta\beta$ of 0.5, 387 CpG island probes that map to 199 genes were found to be differentially methylated in VSCCs compared to normal vulvar tissues with statistical significance. Of these, 195 genes were hyper-methylated and 4 genes were hypo-methylated. Using unsupervised hierarchical clustering, clear clustering of the differentially methylated probes was observed for VSCCs and normal vulvar tissues [Figures

1 and 2]. However, there was more intra-cluster heterogeneity in VSCCs than in normal vulvar tissues. The top 25 hyper-methylated genes based on the $\Delta\beta$ -values are presented in Table 2, and the complete list of the DMGs is presented in supplement 1. The most frequently hyper-methylated genes were ZIC family member 4 (*ZIC4*), coiled-coiled domain containing 181 (*CCDC181* / *C1orf114*), neuroexophilin 1 (*NXP1*), six homeobox 6 (*SIX6*), zinc finger protein interacting with K protein 1 (*ZIK1*), and zinc finger protein 135 (*ZNF135*).

As for the molecular function, the majority of the genes that were hyper-methylated in VSCCs were found to be involved in transcription regulator activity [Figure 3]. The majority of the DMGs were also significantly associated with non-melanoma solid tumors, non-hematologic malignant neoplasms, extracranial solid tumors, and carcinoma. Based on the z-scores, activity of biological process involved in the regulation of organismal death was found to be significantly increased and those involved in the regulation of the quantity of cells were found to be significantly decreased.

3.3 Copy number variations

Both amplifications and deletions of chromosomal regions were present in VSCCs. Amplifications in chromosomes 3, 8, and 9 were most frequently detected; these were present in > 50% of the VSCC samples [Figure 4].

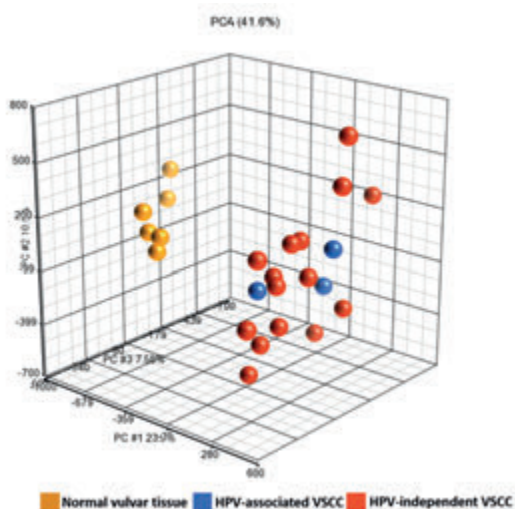


Figure 1: Principal component analysis of the differentially methylated probes show clear clustering for VSCCs and normal vulvar tissues

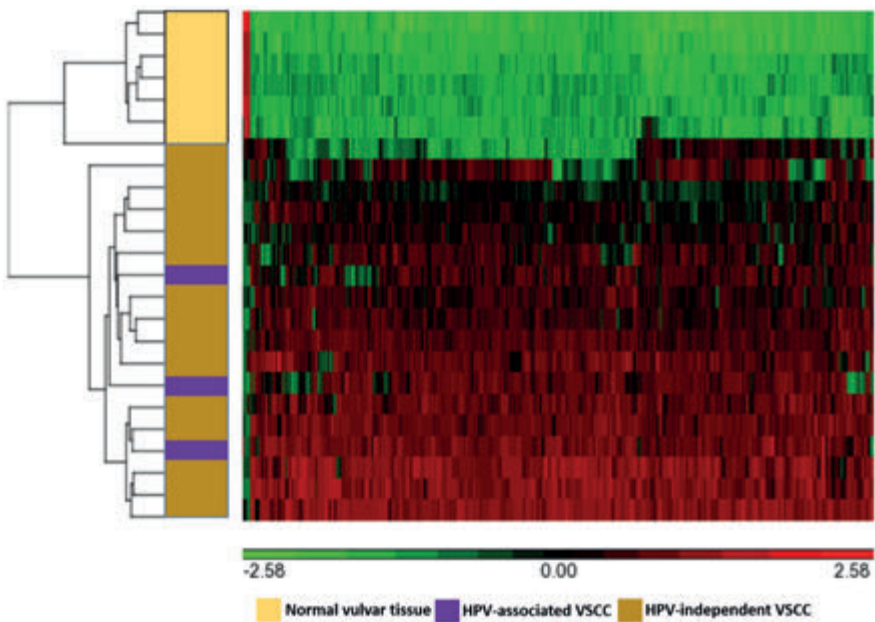


Figure 2: Unsupervised hierarchical clustering of the differentially methylated probes for VSCC and normal vulvar tissues, based on the differences in the β -values ($\Delta\beta$ -values – indicated in the scale). Green represents hypo-methylated and red represents hyper-methylated probes.

8

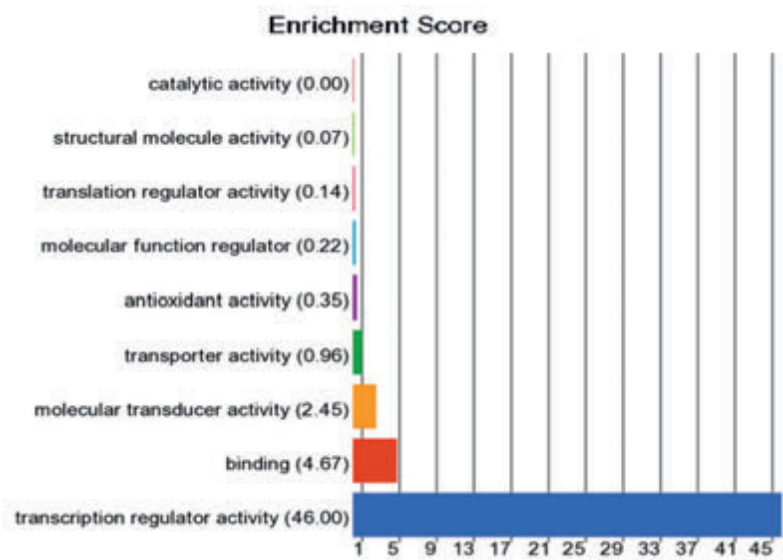


Figure 3: Molecular function of the differentially methylated genes, derived from Gene-Ontology

Table 2: Differentially methylated genes based on $\Delta\beta$ -values (top 25)

Symbol	Entrez Gene Name	$\Delta\beta$ -values	Location	Type(s)
<i>C1QTNF4</i>	C1q and TNF related 4	0.698	Extracellular Space	other
<i>TNR</i>	tenascin R	0.676	Plasma Membrane	other
<i>ZSCAN1</i>	zinc finger and SCAN domain containing 1	0.67	Nucleus	transcription regulator
<i>ZNF135</i>	zinc finger protein 135	0.662	Nucleus	transcription regulator
<i>ZNF471</i>	zinc finger protein 471	0.66	Nucleus	transcription regulator
<i>CCDC8</i>	coiled-coil domain containing 8	0.63	Plasma Membrane	other
<i>TBX3</i>	T-box transcription factor 3	0.626	Nucleus	transcription regulator
<i>LOC728392</i>	uncharacterized LOC728392	0.625	Other	other
<i>SSTR1</i>	somatostatin receptor 1	0.621	Plasma Membrane	G-protein coupled receptor
<i>MARCHF11</i>	membrane associated ring-CH-type finger 11	0.617	Other	other
<i>UNC79</i>	unc-79 homolog, NALCN channel complex subunit	0.615	Other	other
<i>ACSF2</i>	acyl-CoA synthetase family member 2	0.614	Cytoplasm	enzyme
<i>ZIK1</i>	zinc finger protein interacting with K protein 1	0.613	Other	other
<i>KCNC2</i>	potassium voltage-gated channel sub-family C member 2	0.609	Plasma Membrane	ion channel
<i>PABPC5</i>	poly(A) binding protein cytoplasmic 5	0.607	Cytoplasm	other
<i>PPFIA3</i>	PTPRF interacting protein alpha 3	0.604	Plasma Membrane	phosphatase
<i>BARHL2</i>	BarH like homeobox 2	0.603	Nucleus	transcription regulator
<i>BOLL</i>	boule homolog, RNA binding protein	0.603	Cytoplasm	translation regulator
<i>ONECUT2</i>	one cut homeobox 2	0.601	Nucleus	transcription regulator
<i>HAS1</i>	hyaluronan synthase 1	0.6	Plasma Membrane	enzyme

Symbol	Entrez Gene Name	$\Delta\beta$ -values	Location	Type(s)
<i>CMTM2</i>	CKLF like MARVEL trans-membrane domain containing 2	0.596	Extracellular Space	cytokine
<i>ZIC4</i>	Zic family member 4	0.596	Nucleus	transcription regulator
<i>LBX2</i>	ladybird homeobox 2	0.595	Nucleus	transcription regulator
<i>SALL1</i>	spalt like transcription factor 1	0.592	Nucleus	transcription regulator

3.4 Integration of differential methylation and copy number variation analyses

Next, we sought to identify genes that had hyper-methylation in one allele and deletion in another allele. For this, we performed an overlay analysis of all the hyper-methylated probes ($n = 631$; this included both CpG-island and non-CpG-island probes), and genes that were deleted in at least 6 samples of VSCC and not amplified in any sample of VSCC. A set of 5 such genes were identified, namely, amyloid beta precursor like protein 2 (*APLP2*), Rho guanine nucleotide exchange factor 12 (*ARHGEF12*), chondroitin sulfate N-acetylgalactosaminyltransferase 1 (*CSGALNACT1*), glutamate metabotropic receptor 7 (*GRM7*), and PRICKLE2 antisense RNA 1 (*PRICKLE2-AS1*). The ranges of the β -values of these genes in VSCC and in normal vulvar tissues is presented in Figure 5, and their molecular functions and the associated canonical pathways are presented in supplement 2.

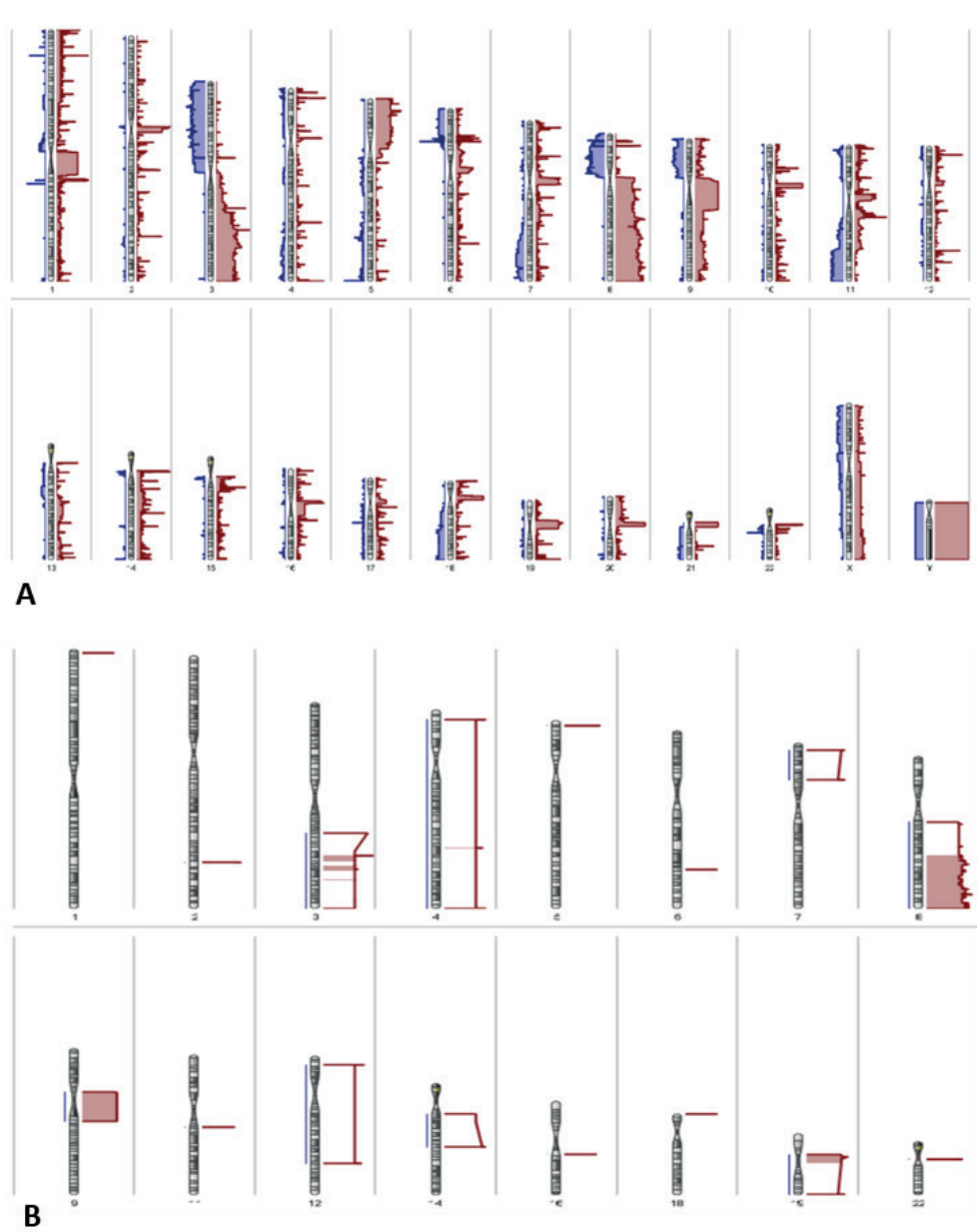


Figure 4: Karyogram depicting the amplifications (red) and deletions (blue) detected in A. all VSCC samples, frequent amplifications in VSCC; B. in > 50% of VSCC samples

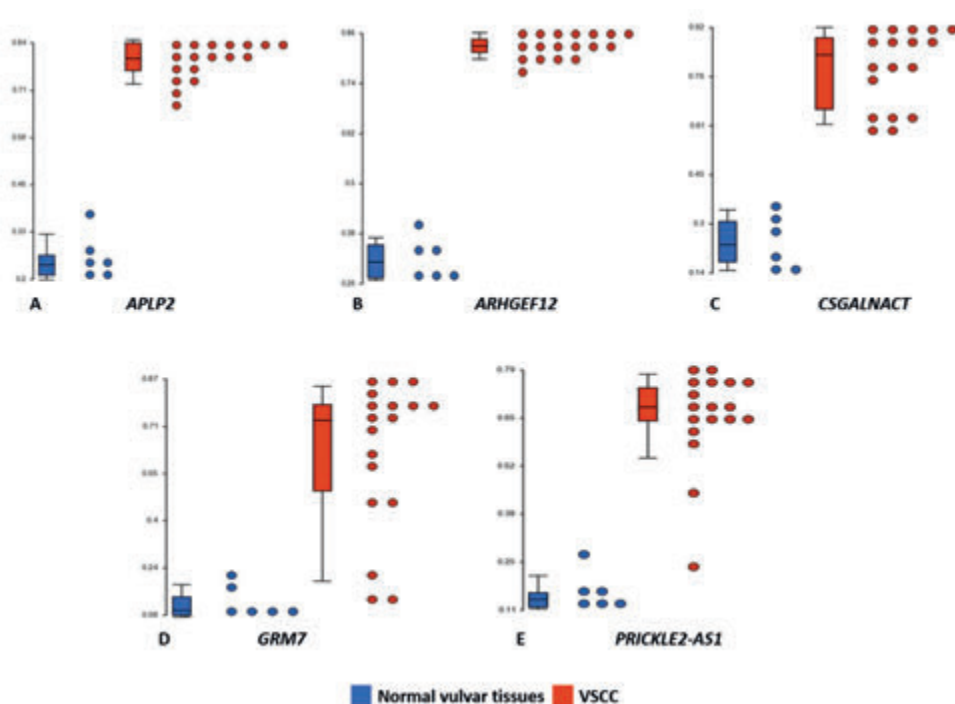


Figure 5: Box-plots depicting the range of β -values in normal vulvar tissues and VSCC, for the 5 genes that are potentially silenced in VSCC.

4. Discussion

In recent years, characterization of the genome and epigenome has provided novel insight into the carcinogenesis of several cancers, and has facilitated diagnosis and tailoring of therapeutic strategies. Genomic changes in the form of somatic mutations can alter gene expression and drive the carcinogenesis. For VSCC and its precursor lesion, vulvar intraepithelial neoplasia (VIN), somatic mutations have been investigated in a number of studies using targeted (amplicon panel based) sequencing, or whole exome sequencing [7,10-12,18,29-33]. However, for comprehensive understanding of the carcinogenesis, characterization of epigenetic changes is also crucial. Unfortunately, for VSCC these changes have been interrogated in only a few studies [34-40].

In this study, we used a high throughput genome-wide methylation array to identify genes that are differentially methylated in VSCC compared to normal

vulvar tissue. DNA-methylation is the most well studied epigenetic mechanism, and is known to play a critical role in repressing gene-expression and maintaining genomic stability. We identified a set of 199 DMGs for VSCC; of these, the majority (n = 194) were hyper-methylated; hyper-methylation is considered to downregulate gene-expression.

Interestingly, on unsupervised hierarchical clustering, distinct clusters of differentially methylated probes were identified for VSCC and for normal vulvar tissue. However, these probes were more heterogeneous for VSCCs than for normal vulvar tissues. Intra-and inter-tumoral heterogeneity in terms of mutational and copy number variation profile of VSCCs have been previously reported [26,33]. Our results indicate the existence of inter-tumoral heterogeneity in the methylation profiles of VSCC. We did not observe any specific clustering corresponding to the HPV-status of VSCC, however, this could be due to the smaller number of HPV-associated VSCCs in this study.

Of the DMGs that were detected for VSCCs, *ZIC1*, *PRDM14*, *SST*, *LHX8*, and *ZNF582* have been previously detected in HPV-associated SCC of the cervix and the anus, and also in their respective precursor lesions [41,42]. We observed that *ZIC1* was hyper-methylated in both subtypes of VSCC, indicating an involvement of this gene in the HPV-independent pathway of VSCC carcinogenesis as well. A recent study reported an association between increasing methylation levels of *ZIC1* and the risk of progression of VIN to VSCC [40]. One of the hyper-methylated DMGs in our study, *DNMT3A*, has been previously detected in VSCC, and was reported to correlate with a risk of recurrence [37].

The majority of the genes that we found to be hyper-methylated in VSCCs are involved in transcription regulator activity, indicating a disruption of this process in VSCC carcinogenesis. We also observed amplifications of chromosomes 3, 8, and 9, to be present in > 50% of the VSCCs; amplification of chromosomes 3 and 8 has also been previously detected in cervical SCC and VSCC [5,41]. Finally, we identified a set of 5 genes that were hyper-methylated, as well as, frequently deleted in VSCC. Of these, somatic mutations of *APLP2* and *ARGHEF12* have been detected in head-and-neck SCC and cervical SCC respectively, and that of *GRM7* and *GALNACT* have been detected in endometrial carcinoma [43-45].

One of the strengths of this exploratory study is the use of a high-throughput genome-wide methylation sequencing array to interrogate the methylation

profiles of VSCC and normal vulvar tissue. The control tissues used in this study were derived from age-matched women with no history of vulvar (pre) malignancies. This offers a unique advantage in that these tissues are unlikely to harbor background molecular changes that may be present in paired normal tissues obtained from patients with cancers, which are often used as controls in molecular studies. However, the lack of such paired normal tissues in this study may also be a potential limitation, as molecular analysis of these tissues could provide insight into the underlying changes in the carcinogenic field. Furthermore, this study was limited by the small sample size, as a result of which heterogeneity in the methylation profiles of VSCCs could not be interrogated by performing sub-group analysis. Recent studies suggest that methylation changes are an early event in VSCC carcinogenesis. Therefore, methylation levels of the DMGs identified in this study in the precursor lesions of VSCC or VIN, could be investigated.

5. Conclusion

We identified a set of DMGs in VSCC in this study with a view to promote hypothesis-generation, and to provide a resource for future investigations to evaluate the diagnostic, prognostic, predictive, or therapeutic potential of these methylation-based biomarkers.

6. References

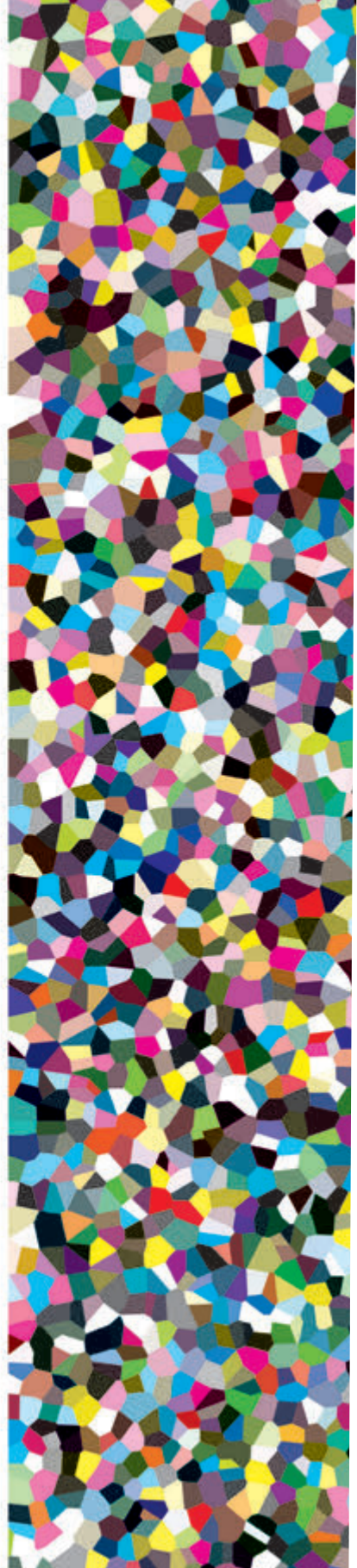
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Supplementary material can be downloaded from this link: <https://drive.google.com/drive/folders/1tL1EPC2TEEPskCemSyHDZ7t30sYKZnyk?usp=sharing>



CHAPTER 9

CAN RADICAL SURGICAL TREATMENT OF THE VULVA BE JUSTIFIED IN THE ABSENCE OF A CONCLUSIVE DIAGNOSIS OF SQUAMOUS CELL CARCINOMA ON BIOPSY? A RETROSPECTIVE 10-YEAR COHORT STUDY

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Abstract

The extent of surgical treatment for vulvar lesions is predominantly guided by the histopathologic diagnosis rendered on the pre-operative biopsy. For premalignant lesions, local excisions are performed, whereas for vulvar squamous cell carcinoma (VSCC), more radical procedures are mandatory. However, even in the absence of a conclusive diagnosis of VSCC on biopsy, the surgeon may opt for a radical excision on grounds of strong clinical suspicion, with a view to avoiding repeat surgeries. We studied a retrospective, 10-year cohort of patients who underwent vulvar excisions, in the absence of a conclusive biopsy diagnosis of VSCC. We aimed to identify the factors predictive of VSCC in these patients, and assess their treatment.

All patients who underwent vulvar excision (2005 – 2016) at Erasmus MC, without a definitive diagnosis of VSCC on the preoperative biopsy were included. Logistic regression analysis was performed to identify the factors predictive of a final diagnosis of VSCC. Surgical treatment was categorized as definitive, incomplete, or over-treatment, based on histopathology of the excision specimen and previous surgical history.

In 57% (64/113) of all included patients, the final diagnosis was VSCC. Higher patient age ($p = 0.03$), and suspicion of VSCC on pre-operative biopsy ($p < 0.001$) were associated with a final diagnosis of VSCC on univariate analysis. Suspicion of VSCC on biopsy was the only significant predictor ($p < 0.001$) on multivariable analysis. For patients with a suspicion of VSCC on biopsy, radical treatment was more frequently performed ($p < 0.001$), which resulted in over-treatment in only 1 case. Where the surgeon had performed a limited excision despite a suspicion of VSCC on biopsy, high patient age, co-morbidities, location of the tumor close to the anus, and history of previous vulvar surgeries were factors which influenced the decision. The treatment administered was definitive for 72%, i.e. additional surgeries were not required; 25% received incomplete treatment and needed additional surgeries, and 3% received over-treatment.

Suspicion of VSCC on biopsy is strongly predictive of a final diagnosis of carcinoma. In our cohort, radical treatment performed on patients with clinical and histopathological suspicion of VSCC resulted in minimal over-treatment, and helped avoid second surgeries

1. Introduction

Vulvar lesions clinically suspected to be vulvar squamous cell carcinoma (VSCC), or its precursor, vulvar intraepithelial neoplasia (VIN), are biopsied by the surgeon, and the subsequent management of the patient is guided by the histopathologic diagnosis on the biopsy. For VIN, patients receive topical treatment, ablative therapy, or limited local excision [1-5]. For VSCC, more extensive excisions are performed, if necessary in combination with groin treatment, i.e. sentinel lymph node (SLN) procedure, or groin lymph node dissection (LND) [6,7].

The diagnosis of VSCC may however be missed on the pre-operative biopsy in 8 – 22% of cases [8-11]. This is often due to sampling error, or in some cases, due to improper orientation or sectioning of the biopsy specimen, which hinder adequate histopathologic assessment. When VSCC is not conclusively diagnosed on the pre-operative biopsy, but the clinical suspicion is sufficiently high, the surgeon may opt to perform a radical excision, with or without groin treatment. Quite often in these cases, VSCC is diagnosed in the excision specimen, and the surgeon manages to spare the patient a repeat surgery in the anatomically complex vulvar region. However, if the excision specimen does not show VSCC, it implies that the patient was over-treated. For these cases, an insight into the specific features that may predict the presence of VSCC could improve clinical decision-making.

This exploratory study was therefore conducted on a cohort of patients who underwent surgical treatment of the vulva, in the absence of a conclusive diagnosis of VSCC on pre-operative biopsy. The aims were to identify the factors that might predict the presence of VSCC, and to assess the surgical treatments performed.

2. Materials and Methods

This retrospective, single-center, cohort-study was conducted at Erasmus MC Cancer Institute, which is a tertiary referral center for gynecologic malignancies. Approval of the Medical Research Ethics Committee was obtained (MEC 2017-134).

2.1 Patient population

All patients who underwent vulvar surgical excisions between January 1, 2005, and January 1, 2016 were identified from the records of the Department of Gynecologic Oncology. Of these, patients who did not have a conclusive diagnosis of VSCC on their pre-operative biopsy(ies) were included. All patient data were anonymized.

If the patient underwent vulvar biopsy followed by an excision more than once, only the first episode was analyzed. Patients with vulvar malignancies other than conventional VSCC, e.g. verrucous carcinoma, basal cell carcinoma, adenocarcinoma, sarcoma, or melanoma, were excluded.

2.2 Data retrieval

Clinical data such as age, diameter and focality of the lesion as noted during clinical examination, history of previous vulvar lesions, treatment details of previous, current, and subsequent episodes, and follow-up, were collected from patient records. Remarks on the treatment rationale, where available, were also recorded.

Pathology data were extracted from the reports of the pre-operative biopsy and the post-operative excision specimens. For VIN, the histopathologic type, i.e. differentiated VIN (dVIN), or high grade squamous intraepithelial lesion (HSIL) was recorded. For VSCC, tumor size, depth of invasion, focality, differentiation grade, and presence of perineural or lymphovascular space invasion were recorded. Status of SLN or LND specimens was noted as positive or negative for metastasis. Slides from all external referral cases had been reviewed by the departmental pathologists before the surgical procedure. All cases where the slides were still available in the departmental archive were reviewed by two pathologists (SDG and PEG) for the purpose of this study. PEG is an experienced gynecologic pathologist.

2.3 Categorization of surgical treatments

The surgical procedures were categorized as:

Local treatment: Wide local excision (WLE), or radical vulvectomy (RV). The aim was to excise with a margin of 5mm for VIN, and 10mm for VSCC, but this was not always achievable.

Groin treatment: SLN procedure, or full groin LND.

Radical treatment: Treatment of both vulva and groin(s), i.e. WLE or RV, with groin treatment.

For each patient, the treatment was categorized as definitive, incomplete, or over-treatment, based on the histopathologic diagnosis of the post-operative specimen, and taking into account the previous history of vulvar surgery, as elaborated below.

Definitive treatment: The surgical procedure was deemed sufficient, and no additional procedures were required, e.g. WLE for VIN, or radical treatment for primary VSCC. For recurrent VSCC, where groin treatment had been conducted previously, WLE with adequate margins was considered definitive.

Incomplete treatment: The surgical procedure was deemed insufficient, and additional procedures were required, e.g. where WLE had been performed, and VSCC stage T1b was diagnosed in the post-operative excision specimen; this meant a subsequent SLN procedure was needed.

Over-treatment: The surgical treatment was deemed to be in excess of what was necessary, e.g. where radical treatment had been performed, and the post-operative excision specimen did not show VSCC

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2.4 Statistical Analyses

Data were analyzed using SPSS Statistics 25 (IBM Corp., Armonk, NY, USA). Descriptive statistic was used for patient characteristics. Independent sample's t-test was used for continuous data, and Chi-squared (χ^2) test for categorical data. Logistic regression analysis was performed to study the effect of several variables on the final diagnosis of VSCC, which was considered as a dichotomous outcome (present or absent). Subgroup analysis of patients with or without a suspicion of VSCC on pre-operative biopsy was also conducted. Two-sided p-value < 0.05 was considered significant.

3. Results

3.1 Characteristics of all included patients

Of 1245 patients who underwent vulvar excisions, 113 met the inclusion criteria. The patients had a mean age of 65.3 years (range 24 – 91 years). The median lesional diameter was 21.5 mm, and the lesion was unifocal in 77% (87/113) of patients [Table 1].

Suspicion of VSCC was present on the pre-operative biopsy in 49% (55/113) of patients [Table 1]. Local treatment was performed for 75% (85/113), and radical treatment for 25% (28/113) of patients. The final diagnosis on post-operative histopathology was VSCC for 57% (64/113), dVIN for 13% (15/113), HSIL for 29% (33/113), and lichenoid changes for 1% (1/113) of patients. Pathology details of the VSCCs are presented in Table 2. The surgical procedure performed was definitive for 72% (81/113), incomplete for 25% (28/113), and over-treatment for 3% (4/113) of patients. The distribution of patients based on their pre-operative biopsies, and subsequent treatment is illustrated in Figure 1.

3.2 Comparison of patients with a final diagnosis of VSCC (n = 64), and without a final diagnosis of VSCC (n = 49)

On univariate analysis, mean age was significantly higher ($p = 0.03$), and suspicion of VSCC on the pre-operative biopsy was significantly more frequent ($p < 0.001$) for patients with VSCC. No other statistically significant difference was found [Table 1]. On multivariable analysis, only suspicion of VSCC in the pre-operative biopsy remained a significant predictor of a final diagnosis of VSCC ($p < 0.001$) [Supplementary Table S1].

Forty patients with VSCC had received local treatment, and a second surgery was required for 25 of these patients. Twenty-four patients with VSCC had received radical treatment, and second surgery was required for 3 of these patients [Figure 1]. Thus, 56% (36/64) of patients with VSCC had received definitive treatment, and 44% (28/64) had received incomplete treatment. Additional surgeries performed for VSCC patients with incomplete treatment included local treatment (WLE) in 6 cases, radical treatment in 11 cases (WLE with SLN procedure in 8 cases, and WLE with LND in 3 cases), and groin treatment in 11 cases (SLN procedure in 8 cases and LND in 3 cases) [Figure 1].

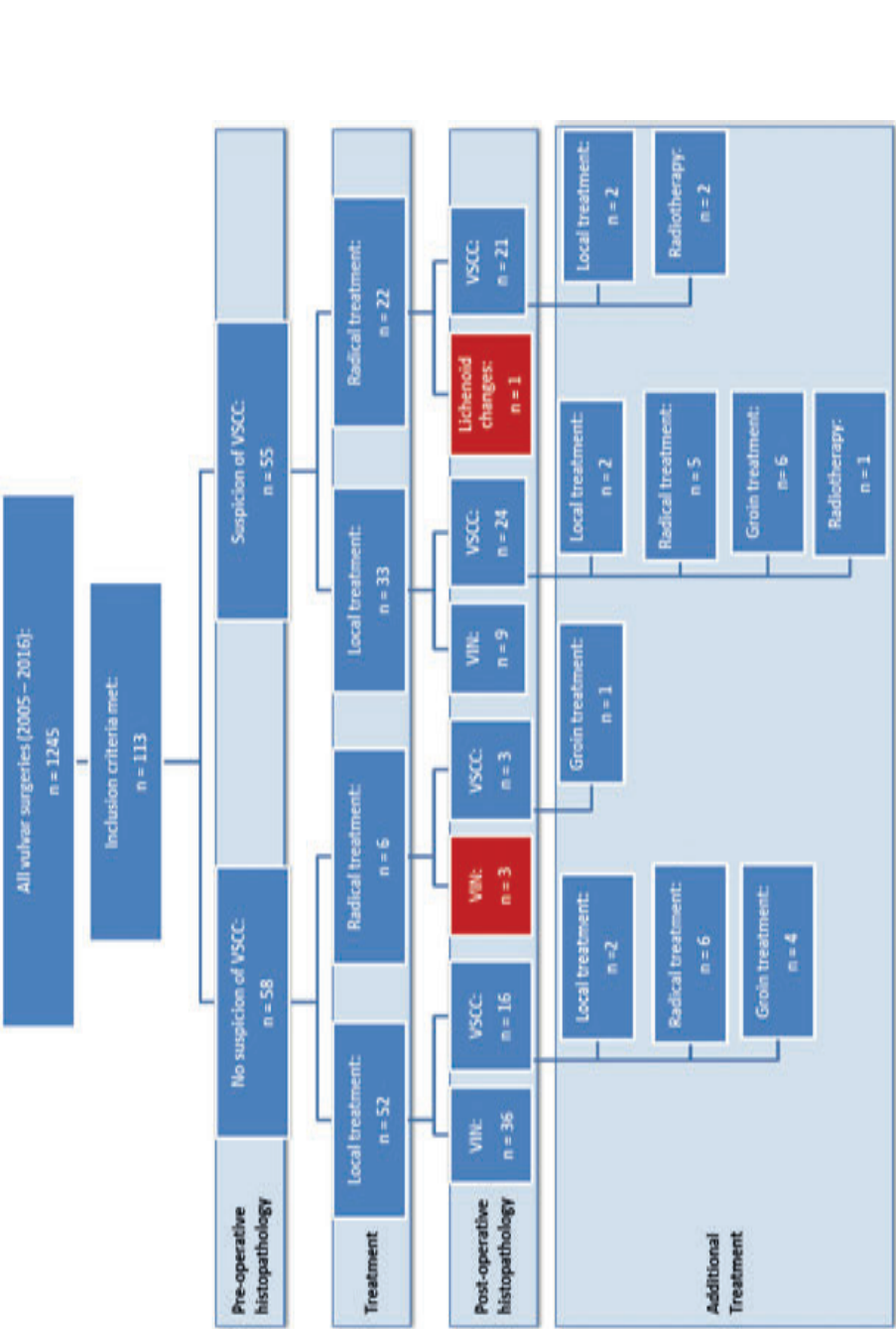


Figure 1. Distribution of patients according to their pre-operative biopsy findings, and treatments received (VIN: vulvar intraepithelial neoplasia, VSCC: vulvar squamous cell carcinoma, boxes in red indicate the cases where there was an over-treatment)

Forty-five patients without VSCC had received local treatment, which was the definitive treatment, and 4 had received radical treatment (WLE with SLN procedure), which was an over-treatment [Figure 1]. For 3 of these patients, the final diagnosis on post-operative histopathology was VIN, and for 1 patient, this was lichenoid changes. Thus, treatment administered was definitive for 92% (45/49), and over-treatment for 8% (4/49) of patients without VSCC.

3.3 Comparison of patients with a suspicion of VSCC (n = 55), and without a suspicion of VSCC (n = 58) in pre-operative biopsy

Suspicion of VSCC was present in the pre-operative biopsy in 49% (55/113) of all included patients. Radical treatment had been performed for 40% (22/55) of patients with a suspicion of VSCC, compared to 10% (6/58) of patients without a suspicion of VSCC ($p < 0.001$) [Table 3]. Radical treatment on patients with a suspicion of VSCC resulted in over-treatment in only 1 case [Figure 1].

The final diagnosis was VSCC in 82% (45/55) of patients with a suspicion, compared to 33% (19/58) of patients without a suspicion of VSCC on biopsy ($p < 0.001$). Suspicion of VSCC had a positive predictive value (PPV) of 82%, sensitivity of 70%, and specificity of 80% for a final diagnosis of VSCC.

Table 1: Patient characteristics

	All (n = 113)	With VSCC (n = 64)	Without VSCC (n = 49)	p-value*
I. Age (in years)				
Mean (95% Confidence Interval)	65.3 (62.3 – 68)	67.9 (64.8 – 71.2)	61.2 (57.1 – 66.4)	0.03
Range	24 – 91	32 – 88	24 – 91	
	Number of patients (percentage)			
II. History of vulvar lesions				
None	65 (58)	39 (61)	26 (53)	0.60
Lichenoid lesions	9 (8)	6 (9)	3 (6)	
VIN	17 (15)	9 (14)	8 (16)	
VSCC	22 (19)	10 (16)	12 (25)	
III. Previous groin treatment				
	19 (17)	9 (14)	10 (20)	0.37
IV. Previous radiotherapy				
	6 (5)	5 (7)	1 (2)	0.18

	All (n = 113)	With VSCC (n = 64)	Without VSCC (n = 49)	p-value*
V. Preoperative clinical examination				
A. Diameter of the lesions				
Median (Range) in mm	21.5 (3 – 70)	25 (3 – 65)	20 (3 – 70)	0.75
< 20 mm	41 (36)	25 (39)	16 (33)	0.51
≥ 20 mm	71 (63)	38 (59)	33 (67)	
Unknown	1 (1)	1 (2)	0 (0)	
B. Focality				
Unifocal	87 (77)	49 (77)	38 (78)	1.00
Multifocal	26 (23)	15 (23)	11 (22)	
VI. Preoperative histopathology				
No suspicion of VSCC	58 (51)	19 (30)	39 (80)	< 0.001
Suspicion of VSCC	55 (49)	45 (70)	10 (20)	
VII. Surgical procedure				
A. Local treatment				
WLE	85 (75)	40 (63)	45 (92)	
B. Radical treatment				
WLE + SLN	19 (17)	15 (23)	4 (8)	< 0.001
WLE + LND	9 (8)	9 (14)	0 (0)	
VIII. Treatment categorization				
Definitive	81 (72)	36 (56)	45 (92)	< 0.01
Incomplete treatment	28 (25)	28 (44)	0 (0)	
Over-treatment	4 (3)	0 (0)	4 (8)	
IX. Additional treatment				
None	82 (73)	33 (52)	49 (100)	0.0001
Local treatment (WLE)	6 (5)	6 (9)	0 (0)	
Radical treatment (WLE + groin treatment)	11 (10)	11 (17)	0 (0)	
Groin treatment only	11 (10)	11 (17)	0 (0)	
Radiotherapy	3 (2)	3 (5)	0 (0)	
X. Follow up				
	Median follow-up duration: 24 months			
No evidence of disease	90 (80)	48 (75)	42 (86)	0.17
Death				
Related to disease	3 (2)	3 (5)	0 (0)	
Unrelated to disease	4 (3)	3 (5)	1 (2)	
Recurrence of disease				
VIN	7 (7)	3 (5)	4 (8)	
VSCC	3 (2)	2 (3)	1 (2)	
Under palliative care	5 (5)	5 (7)	0 (0)	
No follow up	1 (1)	0 (0)	1 (2)	

Table 2: Pathology details of VSCC cases (n = 64)

Tumor characteristics	Number of patients (percentage)
I. Depth of invasion	
< 1 mm	8 (13)
> 1 mm	49 (77)
Unknown	7 (10)
II. Focality	
Unifocal	54 (84)
Multifocal	10 (16)
III. Differentiation grade	
Well	25 (39)
Moderate	33 (52)
Poor	6 (9)
IV. Perineural invasion	
Present	3 (5)
Absent	61 (95)
V. Lymphovascular space invasion	
Present	2 (3)
Suspicion	7 (11)
Absent	55 (86)
VI. Pathology of the groin lymph node (n = 24)	
Positive	5 (21)
Negative	19 (79)

3.4 Treatment rationale

Treatment rationale was not clearly documented for every case, hence statistical analyses could not be performed. Information could be retrieved for the patients with a suspicion of VSCC (n = 55) on biopsy.

Radical treatment was administered to 22 patients; WLE with SLN procedure for 14 patients, and WLE with LND for 8 patients. Radical treatment was chosen on the grounds of strong clinical suspicion, based on the clinical features. This resulted in definitive treatment of 19 patients, incomplete treatment of 2 patients, and over-treatment of 1 patient.

Local treatment (WLE) had been performed for 33 patients. For 7 patients the VSCC was recurrent; a previous SLN had been performed for 1 patient, and LND for 2 patients. Other factors that influenced the choice to abstain from groin treatment were co-morbidity, high patient age, low clinical suspicion of VSCC,

and suspicion of only superficial VSCC, or a verrucous carcinoma. For 3 patients, WLE was chosen because of the proximity of the tumor to the anus, to minimize the risk of sphincter injury. This resulted in definitive treatment of 20 patients, and incomplete treatment of 13 patients.

Table 3: Characteristics of patients with and without a suspicion of VSCC on pre-operative biopsy

Characteristics	Suspicion of VSCC (n = 55)	No suspicion of VSCC (n = 58)	p-value*
I. Age (in years)			
Mean (95% Confidence Interval)	67.9 (64.3 – 71.5)	62.8 (58.7 – 66.9)	0.06
Range	41 – 91	24 – 88	
Number of patients (percentage)			
II. History of vulvar lesions			
None	36 (65)	29 (50)	0.27
Lichenoid lesions	5 (9)	4 (7)	
VIN	6 (11)	11 (19)	
VSCC	8 (15)	14 (24)	
III. Preoperative clinical examination			
A. Diameter of the lesions			
Median (Range) in mm	30 (3 – 65)	20 (3 – 70)	0.16
< 20 mm	20 (36)	21 (36)	0.62
≥ 20 mm	35 (64)	36 (62)	
Unknown	0 (0)	1 (2)	
B. Focality			
Unifocal	43 (78)	44 (76)	0.86
Multifocal	12 (22)	14 (24)	
IV. Surgical procedure			
A. Local treatment (WLE)	33 (60)	52 (90)	
B. Radical treatment			
WLE + SLN procedure	14 (25)	5 (8)	< 0.001
WLE + LND	8 (15)	1 (2)	
V. Post-operative histopathology			
Lichenoid changes	1 (2)	0 (0)	< 0.001
VIN	9 (16)	39 (67)	
VSCC	45 (82)	19 (33)	
VI. Treatment categorization			
Definitive	39 (71)	42 (73)	0.55
Incomplete treatment	15 (27)	13 (23)	
Over-treatment	1 (2)	3 (4)	

Characteristics	Suspicion of VSCC (n = 55)	No suspicion of VSCC (n = 58)	p-value*
VII. Additional treatment			
None	37 (66)	45 (78)	0.34
Local treatment (WLE)	4 (7)	2 (3)	
Radical treatment			
WLE + SLN	3 (5)	5 (8)	
WLE + LND	2 (4)	1 (2)	
Groin treatment only			
SLN	3 (6)	5 (9)	
LND	3 (6)	0 (0)	
Radiotherapy	3 (6)	0 (0)	
VIII. Follow up			
No evidence of disease	43 (78)	47 (81)	0.58
Death			
Related to disease	2 (4)	1 (2)	
Unrelated to disease	1 (2)	3 (4)	
Recurrence of disease			
VIN	3 (5)	4 (7)	
VSCC	2 (4)	1 (2)	
Under palliative care	4 (7)	1 (2)	
No follow up	0 (0)	1 (2)	

4. Discussion

VSCC was diagnosed in the excision specimen in 57% of patients who underwent vulvar excisions in the absence of a conclusive diagnosis of carcinoma on pre-operative biopsy. A final diagnosis of VSCC appeared to be significantly associated with higher age ($p = 0.03$), and suspicion of VSCC on pre-operative biopsy ($p < 0.001$), on univariate analysis. However, on multivariable analysis, suspicion of VSCC remained the only significant predictor ($p < 0.001$).

For patients with a suspicion of VSCC on biopsy ($n = 55$), radical treatment had been performed more frequently, in comparison to patients without a suspicion of VSCC (40% vs. 10%; $p < 0.001$). Despite the lack of a conclusive diagnosis of VSCC, suspicion of the pathologist probably enabled the surgeon to perform radical treatment more frequently in these patients. Where there was both clinical and histopathologic suspicion of VSCC ($n = 22$), radical treatment (WLE with SLN procedure) resulted in over-treatment in only 1 case [Figure 1]. For this patient, the final histopathology showed scar tissue with lichenoid changes; this case may have had a small focus of invasion, which was removed at biopsy. Where the surgeon had performed a limited excision despite a suspicion of

VSCC on biopsy (n = 33), high patient age, co-morbidities, location of the tumor close to the anus, and history of previous vulvar surgeries were factors which influenced the decision.

In contrast to previous reports, no significant association of lesional size or focality with a final diagnosis of VSCC was found. Preti et al. identified an association of larger sized, and multifocal VIN lesions with occult SCC on univariate analysis in their study [12]. On multivariable analysis however, they discovered tumor size to be the confounder [12]. Raised lesions have also been associated with occult invasive carcinoma [8,9]. Missed diagnoses of carcinoma have been reported to be more frequent for perineal, clitoral, and labial lesions [8,9,12]. Similar analyses could not be performed for our cohort as detailed macroscopic descriptions, or the exact anatomical locations of the tumor were not available for all lesions.

For 30% of patients with VSCC in our cohort, there was no suspicion of carcinoma on pre-operative biopsy. Unsuspected invasion is known to be present in 3.2 – 22% of biopsies from high grade VIN [8,10,13]. These figures however, may be biased, as biopsies are more commonly performed for clinically suspicious lesions, and conservative / topical treatment modalities are otherwise frequently administered for HSIL. These results may also be influenced by the number of biopsies taken. To avoid missing small invasive foci, thorough sampling of VIN is of undeniable importance.

On histopathology review, the diagnoses did not change for any case. For biopsies reported as suspicious for VSCC, complex anastomosing epithelial architecture, accompanied by inflammatory infiltrate was often observed (results not presented). In a few cases, an overwhelming VIN lesion masked underlying small nests of invasion, which were not sampled in the biopsy. Accurate judgement of invasion can be hindered by tangential sectioning of the biopsy specimen, or in biopsies from the central part of carcinoma, without adjacent 'normal' epithelium, or underlying stroma. Evenly spaced nests with rounded or bulbous contours, without desmoplastic stromal reaction are more likely to be the result of tangential sectioning [14,15]. True invasion, in contrast, is characterized by single cells, or nests of keratinocytes, with irregular or angulated contours, invading from the basilar epidermis or from elongated rete ridges, occasionally accompanied by desmoplastic stromal reaction, edema, or inflammation [14,15].

To assess its adequacy, the surgical treatment administered was categorized as definitive, incomplete, and over-treatment. Seventy-two percent (81/113) of patients had received definitive treatment; the final diagnosis was VIN for 56%, and VSCC for 44%. Twenty-five percent (28/113) of patients had received incomplete treatment; their final diagnosis was VSCC. For these patients, additional local treatment was needed in 6 cases, radical treatment in 11 cases, and only groin treatment in 11 cases [Figure 1]. There was an over-treatment of 4 patients (3%); the final diagnosis was VIN for 3 patients, and lichenoid changes for 1 patient.

Our findings reflect the fact that in daily clinical practice, treatment decisions are not based entirely on the pre-operative histopathology, but on the surgeon's practical experience and judgement as well. A surgeon's suspicion for VSCC, needless to say, is not a tangible parameter, and we found that it was not always well documented. As a result, this factor could not be used for the analysis. Nevertheless, through this study, we aimed to enhance our understanding of clinical practice for patients where official guidelines are not directly applicable. Management of patients without a conclusive diagnosis of carcinoma on biopsy is not a well-studied area, and available literature is very limited. The high PPV (82%) of suspicion of VSCC on pre-operative biopsy, and the fact that the majority (72%) of patients received definitive treatment, reflects the expertise of both the pathologist and the clinician, and demonstrates the benefits of a referral gynecologic oncology service. However, due to the retrospective nature of the study, some clinical data could not be accessed, and being a single-center study, our results may not be generalizable; these are potential limitations.

5. Conclusion

Suspicion of VSCC on pre-operative histopathology is a significant predictor of a final diagnosis of VSCC. In our cohort, radical treatment performed on patients with strong clinical and histopathologic suspicion of VSCC helped avoid second surgeries and led to minimal over-treatment. In-depth, well-documented clinical notes on treatment rationale, and protocol deviation (where applicable), along with studying similar multi-institutional cohorts could contribute to the development of improved clinico-pathologic algorithms for patient management.

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10



CHAPTER 10

GENERAL DISCUSSION

General Discussion

The aim of this dissertation is to facilitate the pathological diagnosis of squamous epithelial lesions of the vulva by refining the histological diagnostic criteria and exploring biomarkers of potential diagnostic, prognostic, or therapeutic relevance. In the following sections, the main findings of the studies that comprise this dissertation are discussed in the context of current knowledge.

Low-risk human papillomaviruses (LR-HPVs) can produce lesions having the histology of vulvar seborrheic keratosis (VSK)

The association of HPV and lesions having the histology of VSKs has been long debated [1-6]. Since HPV-status of vulvar lesions can be of consequence for determining the appropriate treatment and follow-up, we investigated this association in **chapter 2**. We detected LR-HPVs in the major proportion of a set of lesions diagnosed as VSK, originally as well as upon stringent histology review. It is important to note that none of these lesions showed features of HPV-cytopathy. In some of the HPV-positive VSKs, expression of the viral marker, HPV-protein E4, was observed, which indicated a productive HPV-infection. The presence of HPV in specific lesional cells, e.g. cells expressing E4 and cells in the basal layers of the epithelium where HPV sets up infection, was confirmed by performing laser-capture microdissection based polymerase chain reaction (LCM-PCR). Pathologists therefore need to be aware that the HPV-status of a lesion may not be reliably determined on the basis of histology alone.

Unfortunately, neither of the cellular markers, p16 and MIB-1, were found to be reliable predictors of the HPV-status of these lesions. HPV-positive VSKs showed extensive patchy p16-expression in most cases. However, this pattern of p16-expression may be difficult to distinguish from that occasionally observed in normal vulvar tissue. All HPV-positive VSKs showed MIB-1 positivity, i.e. expression in the upper 2/3rds of the epithelium. However, MIB-1 positivity was also observed in some of the HPV-negative VSKs.

We propose that when diagnosing lesions having the histology of VSKs, particularly in women with a history of HPV-related genital lesions, immunohistochemistry (IHC) with p16 and MIB-1 may be considered. Where p16 shows extensive, patchy expression, and MIB-1 shows increased expression in upper 2/3rds of the epithelium, PCR-testing may be performed to rule out HPV-infection.

Knowledge of vulvar intraepithelial neoplasia (VIN) has advanced, however differentiated VIN (dVIN) remains a diagnostic challenge

Ever since the first report in 1922 by Bowen [7], there has been considerable progress in the knowledge of precursor lesions of vulvar squamous cell carcinoma (VSCC). In **chapter 3**, we presented a summary of the current knowledge in this field, with emphasis on the histology and biomarkers.

A significant development in the field of VSCC was the recognition of its two distinct etiopathological subtypes – HPV-related, and HPV-independent, and their respective precursor lesions, high-grade squamous intraepithelial lesion (HSIL) / usual VIN (uVIN), and differentiated vulvar intraepithelial neoplasia (dVIN) [8-10]. While the diagnosis of HSIL is mostly straight-forward, diagnosing dVIN can be a challenge even for experienced pathologists, due to the similarity in its histological appearance with reactive dermatoses such as lichen sclerosus (LS) [11-15]. As a result, the diagnosis of dVIN has been associated with poor inter-observer agreement [15]. Studies report that although the majority of VSCCs are HPV-independent, dVIN constitutes only 2 – 10% of all VIN diagnoses [13]. Furthermore, a study from the Netherlands reported that of lesions regarded as LS on biopsy and progressing to VSCC, 42% could be re-classified as dVIN on histology review [16]. These reports indicate that dVIN may be under-diagnosed, which has serious clinical consequences, as dVIN is known to progress rapidly to VSCC [17, 18]. Failure to diagnose dVIN therefore signifies missed opportunities for intercepting progression to VSCC.

To improve the diagnostic accuracy for dVIN, the International Society for the Study of Vulvovaginal Diseases (ISSVD) recommends universal use of p53-IHC for cases where dVIN is suspected on clinical or on histological examination [19]. Mutant patterns (overexpression or null-pattern) of p53-expression support a diagnosis of dVIN and help rule out non-dysplastic mimics, which typically show wild-type p53-expression [20-22]. However, recent studies have identified a subcategory of dVIN that does not harbor *TP53* mutations, and therefore shows wild-type p53-expression [19, 21-24]. Therefore, p53-IHC may not inform the diagnosis of dVIN in every case.

To facilitate pathological diagnosis of dVIN, reliable histological criteria and biomarkers (immunohistochemical and molecular) need to be established.

We sought to refine histological diagnostic criteria of dVIN, and explore biomarkers that may aid the diagnosis (chapters 4 – 6). The first step (**chapter 4**) was to identify the most specific histological features of dVIN. For this, we compiled a list of histological features of dVIN from the literature, and recorded their presence or absence in a large retrospective series of dVIN and LS (2010 – 2013). Interestingly, we observed that nuclear atypia, the sine qua non for dVIN diagnosis, could be discerned under low (100X) magnification in only 63% of cases. Among the 15 histological features that were assessed, we identified angulated nuclei, macronucleoli, individual cell keratinization, deep keratinization, and deep squamous eddies to be the most specific for dVIN. These features could also be interpreted with substantial agreement [$\kappa > 0.6$] by two experienced pathologists from our center, who independently reviewed a subset of the slides, blinded to the clinical details. We therefore concluded that these features were ‘most helpful’ for diagnosing dVIN.

Nevertheless, it needed to be determined whether these features can be interpreted with a similar level of agreement in the real-world, i.e. by pathologists with varying levels of experience and from different practice settings, academic or non-academic. Thus, the next step (**chapter 5**) was to assess the agreement in the interpretation of these features among a bi-national, multi-institutional group of pathologists. For this, a selection of slides was prepared from the previously reviewed cohort (**chapter 4**) in a way to include diagnostically straight-forward cases, as well as, cases where the distinction between dVIN and no-dysplasia could be difficult. Nine pathologists from seven centers independently diagnosed these slides as dVIN or no-dysplasia, indicated which features they used and scored their usefulness for the diagnosis of dVIN. The diagnoses rendered by the majority (> 50%) of the participants were taken as the consensus (gold-standard).

Moderate overall agreement ($\kappa = 0.42$) was obtained for the diagnosis of dVIN, which underscores the difficulty of the diagnosis. For the majority of histological features, overall inter-observer agreement was fair (κ : 0.21 – 0.34), while agreement between the participant-pairs ranged from slight ($\kappa = 0.01$) to near-perfect ($\kappa = 0.94$). Based on the proportions of substantial / near-perfect agreement between the participant-pairs, and the ratings of diagnostic usefulness, the most helpful features were parakeratosis, cobblestone appearance, chromatin abnormality, angulated nuclei, atypia discernable under 100X, and altered cellular alignment. Particularly for cases where nuclear atypia

could not be discerned under 100X, we observed that the participants recorded parakeratosis and cobblestone appearance as very useful for diagnosing dVIN.

In this study (**chapter 5**), we also correlated the histological consensus diagnoses with the immunohistochemical expression of p53. p53-IHC patterns showed substantial concordance with the histological consensus diagnoses, which confirms that routine use of this marker can improve the diagnostic accuracy. However, 26% of the slides diagnosed as dVIN by consensus showed wild-type p53-expression, and this highlights the need for additional immunohistochemical markers to aid the diagnosis of dVIN.

As summarized in **chapter 3**, a plethora of immunohistochemical markers has been previously studied for dVIN. However, the majority of these markers have been assessed in only a single study, and / or in a small cohort.

In recent years, expression of cytokeratins (CKs), in particular, CK13 and CK17, has been extensively studied for precursor lesions for oral SCC (OSCC), which, similarly to VSCCs, arises independent of an HPV-infection in most cases [25-28]. As CKs are cell type specific intermediate filament proteins, their expression is altered in abnormalities of cellular differentiation. Apart from OSCC precursors, CK13 and CK17 have also been studied for precursors of esophageal, laryngeal, and cervical SCC [29-31]. CK17 has also been studied for dVIN and precursor lesions of anal SCC [32, 33].

In normal oral mucosa, CK13 is expressed from the basal to the superficial layers [28]. On the other hand, in normal skin (genital / non-genital), CK17 is expressed only in the appendages, and not in the squamous epithelial lining [34, 35]. In premalignant states, CK13-expression is lost and CK17-expression is increased, and the extent of these changes in expression have been reported to correlate with the histological grade of the lesions. Thus, loss of CK13-expression and an increase in CK17-expression has been observed in precursors of oral and cervical SCC [27, 28, 30, 35]. Increased CK17-expression has also been observed in dVIN [32].

In **chapter 4**, we explored the usefulness of CK13 and CK17 to discriminate dVIN from LS. We observed that CK13-expression was lower and CK17-expression was higher in dVIN, than in LS. Among the two markers, CK17 showed better sensitivity and specificity than CK13 for the diagnosis of dVIN. Unlike that reported for OSCC precursors, we did not find any added advantage of using

CK13 and CK17 simultaneously. However, similarly to a previous study on dVIN [32], we found diffuse CK17-expression across full epithelial thickness or in the suprabasal layers to be strongly supportive of a dVIN diagnosis.

Although CK17 showed promise as a diagnostic adjunct for dVIN in our exploratory study (**chapter 4**), we intended to validate these results in an independent cohort of cases. This was performed in the study described in **chapter 6**. In addition, we investigated the usefulness of SRY-box transcription factor 2 (SOX2) in **chapter 6**. SOX2 is a stemness regulator that maintains self-renewal properties of normal and malignant cells, and has been extensively studied for SCC and its precursor lesions in genital and non-genital locations. Expression of SOX2 has also been previously studied in dVIN and VSCC, and increased SOX2-expression has been observed in dVIN. For thorough evaluation of the markers, we compared the expression of CK17 and SOX2 in a retrospective cohort of dVIN, differentiated exophytic vulvar intraepithelial lesion (de-VIL; a novel precursor of *TP53*-wild type HPV-independent VSCC), HSIL, and non-dysplastic vulvar tissues (2014 – 2017). To investigate the usefulness of CK17 and SOX2 as adjunct to the use of p53, we compared the performance (sensitivity, specificity) of these markers with that of p53. In addition, to facilitate the identification of other potential diagnostic markers, we performed next generation targeted sequencing (NGTS) on a subset of dVIN and de-VIL.

Similarly to our exploratory study (**chapter 4**), increased expression of CK17 was observed in dVIN (p53-mutant or p53-wild-type), compared to non-dysplastic vulvar tissue. SOX2-expression was also observed to be higher in dVIN (p53-mutant and p53-wild-type) than in non-dysplastic vulvar tissue. Expression of both CK17 and SOX2 was also higher in de-VIL and HSIL, compared to non-dysplastic vulvar tissue. For both CK17 and SOX2, diffuse, moderate-to-strong expression across full epithelial thickness, or in the basal and suprabasal layers showed high sensitivity and specificity for the diagnosis of dVIN. Among p53, CK17, and SOX2, the highest sensitivity was obtained for SOX2, whereas, the highest specificity was obtained for p53. We therefore believe that CK17 and SOX2, when used in a panel along with p53, can complement the pathological assessment of dVIN. However, CK17 and SOX2, cannot be used to discriminate dVIN from HSIL. Moreover, for discriminating dVIN from non-dysplastic lesions, CK17 and SOX2 should be interpreted in the context of histology and clinical information, and should not be used as the sole diagnostic criterion.

With NGTS, *TP53* and *CDKN2A* mutations were detected most frequently in dVIN, in line with previous reports [12, 24, 36-40]. Interestingly, *TP53* mutations were also detected in de-VIL, and dVIN that showed wild-type-expression on p53-IHC. In de-VILs, we could detect *PIK3CA* mutations, which have been reported to characterize these lesions [41, 42]. However, these were not present in all de-VILs. We observed that a large number of the mutated genes in both dVIN and de-VIL could be mapped to the PI3K/AKT/mTOR pathway [43], indicating a potential involvement of this pathway in HPV-independent VSCC carcinogenesis. Nevertheless, as NGTS was performed on a small number of samples, further investigation in larger cohorts is necessary to determine the clinical significance of these preliminary findings.

Differentially expressed genes (DEGs) and differentially methylated genes (DMGs) provide new insights into VSCC carcinogenesis, and help identify biomarkers of potential significance

Comprehensive molecular characterization of cancer, and comparing the molecular profiles of cancerous and normal tissue from the same anatomical site is essential for the identification of biomarkers of clinical interest. For several cancers, molecular characterization has provided unique insights into the carcinogenesis process, and has enabled personalization of treatment by the identification of targetable molecular changes. Similar progress, unfortunately, has not been possible for VSCC, due to the lack of molecular profiling studies. The mainstay of VSCC treatment remains surgical excision, which is often associated with post-operative morbidity due to the anatomical complexity of the vulvar region. Discovering biomarkers for targeted treatment may expand therapeutic options for VSCC.

In **chapter 7**, we aimed to identify genes that are differentially expressed in VSCC and normal vulva, by analyzing datasets of gene expression microarray from two independent studies, using the latest bioinformatics tools. We further investigated the expression of some of the differentially expressed genes (DEGs) identified thereby, by performing IHC on VSCC, HSIL, dVIN, and normal vulvar tissue.

By performing statistical analysis of microarrays from both datasets, 88 DEGs were identified for HPV-related VSCC that were similarly (up / down) regulated with statistical significance; signal transducer and activator of transcription 1 (STAT1) was one of the upregulated DEGs. For HPV-independent VSCC, 46

DEGs were identified that were similarly (up / down) regulated with statistical significance; nuclear factor IB (NFIB) was one of the downregulated DEGs. We found that the majority of DEGs that were identified for HPV-related VSCC are involved in the immune response, whereas those identified for HPV-independent VSCC are involved in second messenger signaling, which provides support for the dual pathogenesis of VSCC.

We studied the immunohistochemical expression of 2 of the DEGs, NFIB and STAT1 in whole tissue sections of VSCC, dVIN, HSIL, and normal vulvar tissues. We observed a reduced expression of NFIB in dVIN and in both subtypes of VSCC, in comparison with normal vulva. No discernable difference was however observed in the expression of STAT1 in any of these tissue types. NFIB is a transcription factor which has tumor suppressive, as well as, oncogenic potential, and the levels of tissue expression of NFIB has been correlated with adverse prognosis in cervical and head-and-neck SCC. Furthermore, high-confidence proximity interactions have been reported between NFIB and SOX2, increased expression of which was observed in dVIN (**chapter 6**). In view of these observations, we believe that future studies should investigate the role of NFIB in VSCC and its potential as a therapeutic target.

Another key method to identify potential biomarkers is to study the genes that are differentially methylated in cancerous and normal tissue. Methylation is the most widely-studied mechanism of epigenetic modifications, which lead to changes in gene-expression without alterations in DNA sequences. To identify differentially methylated genes (DMGs) in VSCC, we performed genome-wide methylation analysis on a set of VSCC, and normal vulvar tissues from women with no history of vulvar (pre)malignancies (**chapter 8**).

We identified a set of 199 unique genes that were differentially methylated in VSCC, in comparison with normal vulvar tissue, of which 194 were hyper-methylated and 5 were hypomethylated. The majority of the hypermethylated genes are involved in transcription regulator activity, indicating a disruption of this process in VSCC carcinogenesis. We also identified a set of 5 potentially silenced genes in VSCC, as these genes were observed to be hyper-methylated, as well as, frequently deleted. Of these somatic mutations of *APLP2* and *ARGHEF12* have been detected in head-and-neck SCC and cervical SCC respectively, and that of *GRM7* and *GALNACT* have been detected in endometrial carcinoma [44,45].

The control vulvar tissues used in this study were derived from age-matched women with no history of vulvar (pre)malignancies. These tissues are unlikely to harbor background molecular changes that are present in paired normal tissues obtained from patients with cancers, and may provide valuable baseline data for future studies. However, this study was limited by the small sample size, due to which heterogeneity in the methylation profiles of VSCCs could not be interrogated by performing sub-group analysis. As recent studies suggest that methylation changes are an early event in VSCC carcinogenesis, potential of the DMGs identified in this study for the diagnosis of VINs deserve further investigation.

We hope that our results from this study will provide a resource for future investigations to evaluate the diagnostic, prognostic, predictive, or therapeutic potential of these methylation-based biomarkers.

Radical treatment performed on patients with strong clinical and histopathologic suspicion of VSCC helps avoid second surgeries and leads to minimal over-treatment

Although precursor lesions may present the bigger diagnostic challenge, the diagnosis of invasive cancer may also not be straight-forward in every case. Studies report that the diagnosis of VSCC may be missed on the preoperative biopsy in 8 – 22% of cases. These cases complicate treatment decision-making for the surgeon. Where VSCC is not conclusively diagnosed on the pre-operative biopsy, but the clinical suspicion of VSCC is sufficiently high, the surgeon may opt to perform a radical excision. If VSCC is diagnosed in the excision specimen, the surgeon manages to spare the patient a repeat surgery in the anatomically complex vulvar region. However, if the excision specimen does not show VSCC, it implies that the patient was over-treated, and potentially subjected to post-operative complications. For these cases, an insight into the clinical and pathological features that may predict the presence of VSCC could improve clinical decision-making.

To this end, in **chapter 9**, we studied a 10-year retrospective cohort of patients who underwent surgical treatment of the vulva in the absence of a conclusive diagnosis of VSCC on pre-operative biopsy. We also assessed the adequacy of the surgical treatments performed on these patients. We observed that VSCC was diagnosed in the excision specimen in 57% of patients who underwent vulvar excisions in the absence of a conclusive diagnosis of carcinoma on pre-operative

biopsy. For patients with a suspicion of VSCC on biopsy, radical vulvar excision with sentinel lymph node procedure had been performed more frequently in comparison to patients without a suspicion of VSCC ($p < 0.001$). On univariate analysis, we found a final diagnosis of VSCC to be significantly associated with higher age ($p = 0.03$), and suspicion of VSCC on pre-operative biopsy ($p < 0.001$). However, on multivariable analysis, suspicion of VSCC remained the only significant predictor ($p < 0.001$). Where there was both clinical and histopathologic suspicion of VSCC, radical treatment resulted in over-treatment in only 1 case. The majority (72%) of the patients in the cohort had received definitive treatment, i.e. no second surgeries were required.

Our findings reflect the fact that in daily clinical practice, treatment decisions are not based entirely on the pre-operative histopathology, but also on the surgeon's experience. A surgeon's suspicion for VSCC, needless to say, is not a tangible parameter, and we found that it was not always well documented. As a result, this factor could not be used for the analysis. Nevertheless, this study aimed to enhance our understanding of clinical practice for patients where official guidelines are not directly applicable. Our findings highlight that to improve clinico-pathological algorithms for patient management, in-depth, well-documented clinical notes on treatment rationale, and protocol deviation (where applicable) are essential.

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CHAPTER 11

SUMMARY AND FUTURE PERSPECTIVES
NEDERLANDSE SAMENVATTING

Summary and Future perspectives

The majority of lesions affecting the vulva arise from its squamous epithelial lining. This dissertation addresses pathological perspectives of benign, pre-malignant, as well as malignant squamous epithelial lesions of the vulva.

In **chapter 2**, we observed that LR-HPVs can produce lesions that histologically resemble vulvar seborrheic keratosis (VSK) and show no features of HPV-cytopathy. To establish the HPV-association, we performed HPV-DNA polymerase chain reaction, both whole-tissue section-based and laser capture microdissection-based. We also studied immunohistochemical expression of viral (HPV-protein E4) and cellular markers (p16 and MIB1) in these lesions. However, crucial information on HPV-integration in the host genome may be obtained by performing RNA-in situ hybridization. The novel technology RNAscope® may be useful in this regard as it can detect LR-HPVs, and have been shown to have higher sensitivity and specificity than conventional in-situ hybridization techniques. Furthermore, the follow-up of larger cohorts of these lesions should be studied to better characterize their natural history.

In **chapters 4 – 6**, we aimed to refine histological diagnostic criteria of differentiated vulvar intraepithelial neoplasia (dVIN), the precursor lesion of HPV-independent vulvar squamous cell carcinoma (VSCC). We identified a set of histological features of dVIN that could be reproducibly identified by pathologists from the same tertiary center, as well as by pathologists from different academic and non-academic centers. We observed a suboptimal level of agreement for the diagnosis of dVIN, which indicates that dVIN remains a challenging diagnosis even 5 decades after its first recognition. We hope that awareness of these features and regular inter-disciplinary communication will help pathologists accurately diagnose dVIN. In addition, we identified that increased expression of cytokeratin 17 (CK17) and SRY-box 2 (SOX2) correlate strongly with the diagnosis of dVIN, and inferred that both markers when used in a panel along with p53 can complement the pathological assessment of dVIN. Increased expression of CK17 and SOX2 should also be helpful in confirming the diagnosis of the novel entity, differentiated exophytic vulvar intraepithelial lesion (de-VIL). However, we also encountered a number of non-dysplastic lesions that showed increased CK17 and SOX2 expression, whereas, a number of dVIN showed minimal CK17 and SOX2 expression. Therefore, further studies should investigate whether increased CK17 and SOX2 expression in non-dysplastic lesions is associated with

molecular perturbations related to early pre-neoplastic changes that precede histological manifestation.

In this dissertation, although we investigated the agreement in the interpretation of histological features, that for immunohistochemical markers was not assessed. Immunohistochemical markers are the most commonly used ancillary tools in any diagnostic laboratory, and reproducibility in their assessment is as crucial as that for the histological features. As p53 is the most widely used marker for dVIN, a study to measure agreement in the assessment, and to determine how p53 immunohistochemistry influences the decision-making of pathologists for discriminating dVIN from non-dysplastic lesions is underway.

With increasing advancements in the study of artificial intelligence, the potential for developing machine-learning based algorithms for facilitating dVIN diagnosis needs to be urgently explored. The set of slides used in **chapter 5**, that were judged as dVIN by consensus would serve as an excellent training set for this purpose.

Comprehensive molecular characterization of VSCC and its precursor lesions is yet another area of unmet need. Investigation into the genomic profile (next generation targeted sequencing) and epigenomic profile (genome-wide methylation sequencing) of vulvar (pre)cancers and the tissue expression of differentially expressed genes, performed in studies described in **chapter 6 – 8** were exploratory and hypothesis-generating in nature. These results deserve further exploration in larger, multi-centric cohorts.

Finally, **chapter 9** investigates the clinico-pathological factors that may predict the presence of VSCC in patients where a definitive diagnosis of VSCC could not be made on pre-operative biopsy. We identified suspicion of VSCC on pre-operative histopathology to be a significant predictor of a final diagnosis of VSCC. This study highlights the importance of documentation of clinical and histological suspicion of VSCC, and intends to enhance our understanding of clinical practice for patients where official guidelines are not directly applicable.

Nederlandse samenvatting

De meerderheid van de vulvair laesies ontstaat in de bekledende plaveiselepitheel. Dit proefschrift gaat over de pathologische aspecten van benigne, pre-maligne, en ook maligne plaveiselcel laesies van de vulva.

In **hoofdstuk 2** constateren we dat laag-risico (LR)-HPV's laesies kunnen produceren die histologisch kunnen lijken op vulvair verruca seborrhoica zonder kenmerken van HPV-cytopathie. Om de HPV-associatie vast te stellen, werden zowel de gehele weefselsecties en ook specifieke laesionale gebieden doorsneden met laser-capture met HPV-DNA-polymerase chain reaction getest. Tevens bestudeerden we immunohistochemische expressie van virale marker (HPV-proteïne E4) en cellulaire marker's (p16 en MIB1) in deze laesies. Cruciale informatie over HPV-integratie in het host-genome kan daarbij worden verkregen als RNA-in situ hybridisatie wordt getest. De nieuwe technologie RNAscope® kan in dit opzicht nuttig zijn, omdat deze technologie LR-HPV's kan detecteren en gevoeliger en specifiek is dan de traditionele in-situ hybridisatie technieken. Bovendien zou de follow-up van grotere cohorten van deze laesies moeten worden bestudeerd om hun natuurlijke ontwikkeling beter te karakteriseren.

In de **hoofdstukken 4 – 6** probeerden we histologische diagnostische criteria van het gedifferentieerde vulvair intra-epitheliale neoplasie (dVIN), de precursor laesie van HPV-ongereleerd vulvair plaveiselcelcarcinoom (VPCC), te verfijnen. We identificeerden de histologische kenmerken van dVIN die reproduceerbaar konden worden geïdentificeerd door pathologen uit hetzelfde tertiaire centrum, evenals door pathologen uit verschillende academische en niet-academische centra. De suboptimale reproducibiliteit voor de diagnose van dVIN stelt dat dVIN zelfs 5 decennia na de eerste herkenning een uitdagende diagnose blijft. We hopen dat door een groter bewustzijn van deze kenmerken en regelmatige interdisciplinaire communicatie pathologen dVIN beter kunnen diagnosticeren. Bovendien hebben we vastgesteld dat verhoogde expressie van cytokeratine 17 (CK17) en SRY-box 2 (SOX2) sterk correleert met de diagnose van dVIN, en concludeerden dat beide markers bij gebruik in een panel, samen met p53, de beoordeling van dVIN kunnen verbeteren. Verhoogde expressie van CK17 en SOX2 zou ook nuttig kunnen zijn bij het bevestigen van de diagnose van een nieuwe entiteit, genaamd, gedifferentieerde exofytische vulvair intra-epitheliale laesie (de-VIL). Bij ons onderzoek kwamen we echter ook een aantal niet-dysplastische laesies tegen die verhoogde CK17- en SOX2-expressie toonden, en

een aantal dVIN die lagere CK17- en SOX2-expressie toonden. Daarom zouden verdere studies moeten onderzoeken of verhoogde CK17- en SOX2-expressie in niet-dysplastische laesies geassocieerd is met moleculaire verstoringen gerelateerd aan vroege pre-neoplastische veranderingen die voorafgaan aan de histologische manifestatie.

Hoewel we in dit proefschrift de reproducibiliteit in de interpretatie van histologische kenmerken hebben onderzocht, werd dat voor immunohistochemische markers niet beoordeeld. Immunohistochemische markers zijn de meest gebruikte hulpmiddelen in elk diagnostisch laboratorium en reproduceerbaarheid bij hun beoordeling is net zo cruciaal als die voor de histologische kenmerken. Aangezien p53 de meest gebruikte marker is voor dVIN, is er een onderzoek gaande om reproducibiliteit in de beoordeling te meten en om te bepalen hoe p53-immunohistochemie de besluitvorming van pathologen beïnvloedt om dVIN te onderscheiden van niet-dysplastische laesies.

Met de toenemende vooruitgang in de studie van kunstmatige intelligentie, moet het potentieel voor het ontwikkelen van op machine-learning gebaseerde algoritmen voor het vergemakkelijken van dVIN-diagnose dringend worden onderzocht. De coupes die in **hoofdstuk 5** bij consensus als dVIN werden beoordeeld, zou voor dit doeleinde een uitstekende trainingsset zijn.

Comprehensieve moleculaire karakterisering van VPCC en zijn precursorlaesies is nog een onderontwikkeld gebied. Onderzoek naar het genomische profiel (next generation targeted sequencing) en epigenomische profiel (genome-wide methylatieanalyse) van vulvaire (pre)kankers en de weefselexpressie van differentially expressed genen, uitgevoerd in de studies beschreven in **hoofdstukken 6 – 8**, was verkennend en hypothesegenererend. Deze resultaten verdienen verdere onderzoek in grotere, multi-centrische cohorten.

In **hoofdstuk 9** beschrijven we de klinisch-pathologische factoren die de aanwezigheid van VPCC kunnen voorspellen in patiënten bij wie geen definitieve diagnose VPCC was gesteld op basis van hun preoperatieve biopsie. We identificeerden het vermoeden van VPCC op preoperatieve histopathologie als een significante voorspeller van een definitieve diagnose van VPCC. Deze studie benadrukt het belang van het documenteren van klinische en histologische verdenking van VSCC, en is bedoeld om ons begrip van de klinische praktijk voor patiënten waar officiële richtlijnen niet direct van toepassing zijn, te verbeteren.

APPENDIX

APPENDIX

AFFILIATIONS OF CO-AUTHORS

PHD PORTFOLIO

LIST OF PUBLICATIONS

ACKNOWLEDGEMENT

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PhD portfolio

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PhD training		
<i>Courses and Workshops</i>	Year	ECTS
CC02 Biostatistical Methods I: Basic Principles	2017	5.7
Scientific Integrity	2017	0.3
Biomedical Research Techniques	2017	1.5
Basic Course on 'R'	2017	2.0
Basic Introduction Course on SPSS	2017	1.0
Survival Analysis	2018	0.6
Microscopic Image Analysis: From Theory to Practice	2018	0.8
Bayesian Statistics and JASP	2018	0.6
UCSC Gene Browser workshop (Basic/Adv.)	2018	0.7
NCBI & other open source software	2018	1.0
Epigenetic Regulation in Health and Disease	2018	0.8
Introduction in GraphPad Prism Version 7	2018	0.3
Programming with Python	2019	1.0
Presenting Skills for Junior Researchers	2019	1.0
NGS for Clinical Genetics	2019	1.0
SNP Course: SNPs and Human Diseases	2019	2.0
International Society for the Study of Vulvovaginal Diseases Post-graduate course	2019	2.0
English Biomedical Writing and Communication	2020	3.0
Machine Learning using Python	2020	0.8
<i>Language courses</i>		
Dutch A1 – B1.2	2017 – 2019	12.5
<i>Oral presentations</i>		
Jonge Onderzoekersdag, Rotterdam	2017	1.0
Maastricht Pathology, Maastricht (presented by co-author)	2018	1.0
European College for the Study of Vulvovaginal Diseases, Copenhagen	2018	1.0
Erasmus MC Cancer Institute Research Day, Rotterdam	2018	1.0
European Congress of Pathology, Nice (presented by co-author)	2019	1.0
International Society for the Study of Vulvovaginal Diseases World Congress, Turin	2019	2.0
ACE meeting, Department of Gynecologic Oncology, Rotterdam	2019	1.0
European Congress of Pathology, online meeting	2020	1.0
<i>Poster presentations</i>		
Pathologendagen, Ede	2017	1.0

European Congress of Pathology, Amsterdam	2017	1.0
Joint Meeting of the Royal Society of Medicine & the Pathological Society of Great Britain & Ireland, London	2018	1.0
European Congress of Pathology, Bilbao (presented by co-author)	2018	1.0
International Society for the Study of Vulvovaginal Diseases World Congress, Turin	2019	2.0
International Papillomavirus Conference, online meeting (presented by co-author)	2020	1.0
European Congress of Pathology, online meeting	2020	1.0
Teaching activities		
VO microscopische onderwijs	2017 – 2020	4.0
Other activities		
Member of the Communications Committee of International Society for the Study of Vulvovaginal Diseases		
Member of PhD committee of MolMed, Erasmus MC		
Awards		
Cambridge University Press Poster Prize, Joint Meeting of the Royal Society of Medicine & the Pathological Society of Great Britain & Ireland London	2018	
Jonge Onderzoeker Prijs, Nederlandse Vereniging Voor Vulvar Pathologie (1000 €)	2017	

List of publications

Included in this dissertation

1. **Dasgupta S**, Koljenović S, van den Bosch TPP, et al. Evaluation of Immunohistochemical Markers, CK17 and SOX2, as Adjuncts to p53 for the Diagnosis of Differentiated Vulvar Intraepithelial Neoplasia (dVIN). *Pharmaceuticals* 2021; 14, 324. doi: 10.3390/ph14040324
2. **Dasgupta S**, de Jonge E, Van Bockstal MR, et al. Histological interpretation of differentiated vulvar intraepithelial neoplasia (dVIN) remains challenging—observations from a bi-national ring-study. *Virchows Arch* 2021. doi: 10.1007/s00428-021-03070-0
3. **Dasgupta S**, Ewing-Graham PC, van den Bosch TPP, et al. Nuclear factor IB is downregulated in vulvar squamous cell carcinoma (VSCC) – unravelling differentially expressed genes of VSCC through analyses of gene expression datasets. *Oncol Lett* 21: 381, 2021. doi: 10.3892/ol.2021.12642
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Not included in this dissertation

1. Phanthunane C, Wijers R, de Herdt M, Langeveld TPM, Koljenović S, **Dasgupta S**, et al. B-cell clusters at the invasive margin associate with longer survival in early-stage oral-tongue cancer patients. *Oncoimmunology* 2021;10:1. doi: 10.1080/2162402X.2021.1882743.
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4. **Dasgupta S**, Ewing-Graham PC, Groenendijk FH, et al. Granular dot-like staining with MLH1 immunohistochemistry is a clone-dependent artefact. *Pathol Res Pract* 2020;216:152581. doi: 10.1016/j.prp.2019.152581.
5. Blok F, **Dasgupta S**, Dinjens WNM, et al. Retrospective study of a 16 year cohort of BRCA1 and BRCA2 carriers presenting for RRSO: prevalence of invasive and in-situ carcinoma, with follow-up. *Gynecol Oncol* 2019; 153:326-44. doi: 10.1016/j.ygyno.2019.03.003.

Conference abstracts

1. **Dasgupta S**, van Eersel R, Morrel B et al. Possible etiologic role of human papillomavirus in vulvar seborrheic keratosis: a study combining whole tissue section-PCR, immunohistochemistry (p16 and e4), and laser capture microdissection-PCR. **Virchows Arch** 2020, supplement issue.
2. **Dasgupta S**, de Jonge E, Alcalá LS, et al. Inter-observer variability in differentiated vulvar intraepithelial neoplasia. **Virchows Arch** 2020, supplement issue
3. **Dasgupta S**, Ewing-Graham PC, Swagemakers SMA, et al. Precursor Lesions of Vulvar Squamous Cell Carcinoma – Systematic Review And Insights From Gene Expression Omnibus Analysis. **J Lower Gen Tract Dis** 2019, supplement issue.
4. Jonker L, **Dasgupta S**, Ewing-Graham PC, et al. Surgical Treatment of The Vulva in The Absence of A Conclusive Diagnosis Of Squamous Cell Carcinoma, Data From A 10 Year Cohort Study. **J Lower Gen Tract Dis** 2019, supplement issue.
5. **Dasgupta S**, Koljenović S, van Doorn HC, et al. Histological Spectrum of Differentiated Vulvar Intraepithelial Neoplasia And Exploration Of Cytokeratin 17 And SOX 2 Immunohistochemistry As Potential Diagnostic Adjuncts. **J Lower Gen Tract Dis** 2019, supplement issue.
6. **Dasgupta S**, Ewing-Graham PC, de Water VR, et al. Recognising Differentiated Dysplasia in the Head and Neck-Morphological Parameters and Correlation with Cytokeratin 13 and Cytokeratin 17 Staining. **J Pathol** 2018, supplement issue.
7. **Dasgupta S**, Ewing-Graham PC, Noordhoek Hegt V, et al. Improving reproducibility of diagnosis of differentiated Vulvar Intraepithelial Neoplasia (dVIN): Morphological features and immunohistochemistry (cytokeratin 13 and 17). **Virchows Arch** 2017, supplement issue.

Acknowledgement

Author biography

Shatavisha Dasgupta was born in Belghoria, and grew up in Kolkata in West Bengal, India. In 2004, she completed her high-school education with distinction (higher secondary examination). In the same year, she started her medical education at Radha Gobinda Kar Medical College and Hospital in Kolkata, under the West Bengal University of Health Sciences. She received her bachelor in Medicine and Surgery (MBBS) degree in 2010. During her under-graduate medical education, she received honors marks in Physiology, Biochemistry, Preventive and Social Medicine, and Otorhinolaryngology.

In 2011, she started her post-graduate medical education in Pathology at Institute of Post-graduate Medical Education and Research and Seth Sukhlal Karnani Memorial Hospital in Kolkata, under the West Bengal University of Health Sciences. She received her Doctor of Medicine (MD) degree in 2014, based on the results of the MD Pathology Final Examinations and evaluation of her dissertation entitled 'Clinico-Pathological Study of Unhealthy Cervix in Peri-Menopausal Women'. Between 2014 – 2016, she worked as a Pathologist at the Department of Pathology of K.P.C. Medical College and Hospital, Kolkata.

In 2016, Shatavisha joined the department of Pathology, Erasmus MC as a visiting researcher. From 2017 – 2021, she has been working on her PhD project on squamous epithelial lesions of the vulva. During her PhD-tenure, she has also been involved in research projects related to tubo-ovarian pathology, head and neck pathology and methylation profiling. In her free time, Shatavisha enjoys reading, watching movies, painting, and board games.

