“Differentiated Vulvar Intraepithelial Neoplasia and Lichen Sclerosus-like Lesions in HPV-associated Squamous Cell Carcinomas of the Vulva”

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INTRODUCTION

It is widely accepted that most vulvar squamous cell carcinomas (VSCC) originate from intraepithelial lesions that precede the development of VSCC by a variable period of time \(^1\)–\(^3\). Each type of VSCC, i.e., human papillomavirus (HPV)-associated and HPV-independent, not only has particular histologic features but is also associated with a distinct type of intraepithelial lesion \(^3\).

Thus, HPV-associated VSCCs are usually of basaloid, warty or non-keratinizing subtype and arise from high-grade intraepithelial lesions, also referred to as vulvar intraepithelial neoplasia of usual type (HSIL/uVIN), which have basaoid or warty features. In contrast, HPV-independent VSCCs, which are usually of a keratinizing subtype, derive from a premalignant lesion named differentiated vulvar intraepithelial neoplasia (dVIN) and are frequently associated with chronic inflammatory lesions, especially lichen sclerosus (LS) \(^1\),\(^2\). However, this correlation between histological features and HPV involvement is not always the rule, and there is increasing evidence of a frequent overlap between HPV status and the histological characteristics of VSCC \(^3\)–\(^6\). Thus, the presence of keratinizing, basaloid, warty features or koilocytotic changes is highly unreliable to classify the tumor as HPV-associated or HPV-independent \(^5\). The problem is further stressed by the occasional overlap between the premalignant lesions, as in a small percentage of cases the precursors of HPV-independent VSCC may closely simulate HSIL/uVIN \(^7\),\(^8\). A recent meta-analysis has estimated that the pooled prevalence of HPV-related dVIN-like lesions is about 2\% \(^9\). However, no study has specifically addressed the description of these dVIN-like intraepithelial lesions in HPV-associated VSCC.
In this study, we aimed to evaluate adjacent intraepithelial lesions in a large series of 326 VSCC positive for DNA HPV, focusing on unusual histological patterns such as intraepithelial lesions resembling dVIN and/or LS, the skin lesions typically related to HPV-independent VSCC. We analyzed their correlation with the HPV DNA genotype, p16 immunohistochemistry, and HPV mRNA.

**MATERIAL AND METHODS**

**Case selection**

We reviewed a series of 1709 invasive VSCCs included in the VVAP study (International Survey on HPV prevalence and type distribution in vulvar, vaginal, anal, penile neoplasia) coordinated by the Catalan Institute of Oncology (ICO, Barcelona-Spain) in collaboration with DDL Diagnostics Laboratory (Rijswijk, the Netherlands).

We selected all cases fulfilling the following inclusion criteria: (1) invasive squamous carcinoma identified in the block; (2) adequate material for histological analysis, HPV detection and typing, and p16 immunohistochemical staining; (3) a positive result for HPV detection by polymerase chain reaction (PCR); and (4) the presence at least 1 cm of skin adjacent to the invasive tumor. The study was approved by the local and ICO Ethics Committees. Three hundred twenty-six cases fulfilled the inclusion criteria.

**Tissue preparation, nucleic-acid isolation and HPV DNA detection**

DNA extraction was performed on whole sections of formalin-fixed paraffin-embedded (FFPE) tissue from surgical specimens or vulvar biopsies as
previously described\(^5,10\). Sectioning and sample preparation were carried out with the highest safety measures to avoid cross-contamination. Processing and pathology diagnosis were done by the ICO laboratory.

HPV DNA detection was performed using SPF10 PCR, DEIA and the LiPA25 system (version 1, Laboratory Biomedical Products, Rijswijk, The Netherlands) as previously described\(^5,10\). Each run contained negative and positive controls to monitor the efficiency of DNA isolation, PCR amplification, hybridization, and genotyping procedures.

**E6*I mRNA reverse transcription (RT-)PCR**

Extraction and HPV mRNA detection from tissue ribbons were performed as previously described\(^5,11\). For each case, HPV type-specific E6*I mRNA RT-PCR assays were performed for the HPV type(s) determined previously by genotyping and for a cellular ubiquitin C gene as a control for tissue quality. HPV E6*I mRNA assays were developed for the following HPV types: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 70, 73, and 82. A second assay was performed to assess the presence of HPV16 E6*I mRNA in all cases, irrespective of the HPV DNA result.

HPV mRNA extraction and detection was not performed in 22 cases in which low-risk or undetermined HPV types were detected. Thus, HPV mRNA results were available in 304 out of the 326 cases.

**p16 immunohistochemistry**

All tumors were stained with p16 monoclonal antibody using the CINtec Histology Kit (clone E6H4; Roche-mtm-Laboratories, Heidelberg, Germany)\(^12\).
Only cases showing strong and diffuse block staining of the basal and suprabasal layers were considered as positive (p16 upregulation)\textsuperscript{13}. The p16 staining was evaluated independently in the invasive tumor, in the intraepithelial lesion/s and in the normal skin.

**Classification of the lesions as conclusively and non-conclusively associated with HPV**

A case was classified as conclusively associated with HPV when it was positive for HPV DNA in the invasive tumor and had either a positive staining for p16 immunohistochemistry, was positive for HPV mRNA or both. Non-conclusive association with HPV was defined as the identification of HPV DNA in the invasive tumor with negative staining for p16 immunohistochemistry and a negative result for HPV mRNA. Tumors in which only a low-risk or an undetermined HPV type were identified were analyzed independently, as HPV mRNA assays were not developed for these viruses.

**Histological evaluation**

A single histological slide of each case was available for review. Histological evaluation was blind to HPV DNA and genotyping, HPV mRNA, and p16 immunohistochemistry results. Invasive squamous carcinoma was confirmed in all the 326 cases. The VSCC were classified into keratinizing, basaloid, warty or non-keratinizing subtypes following the WHO 2014 classification\textsuperscript{14}.

The squamous epithelium adjacent to the neoplasms was carefully evaluated in search of associated pathology. In order to establish the diagnosis of intraepithelial lesion, the lesion had to be present at least 10 mm away from
the invasive carcinoma to rule out possible peripheral intraepithelial extension simulating HSIL/uVIN or dVIN. The adjacent HSIL/uVIN was classified as a basaloid or warty subtype. Briefly, the basaloid type HSIL/uVIN was characterized by small atypical cells throughout the whole epidermis with a striking architectural disarray 2. Warty type HSIL/uVIN was diagnosed when the epidermis had wide and deep rete ridges, clear pleomorphism and easily recognizable koilocytotic-like changes suggestive of HPV infection 3. dVIN-like lesion was defined by the presence of atypical keratinocytes limited to the basal and parabasal layers in the context of a fully differentiated epithelium 3. LS-like lesions were diagnosed in cases where homogenization of the collagen was clearly evident, alongside other features described elsewhere 1. The adjacent skin was considered normal in the absence of HSIL/uVIN, dVIN-like lesions, and/or LS-like changes.

RESULTS

The adjacent skin was normal in 121/326 VSCC (37.1%), whereas 205/326 (62.9%) showed at least one intraepithelial precursor lesion in the adjacent skin. HSIL/uVIN (warty or basaloid type) was observed as the only precursor lesion in 191 out of 326 tumors (58.6%). Unusual intraepithelial lesions, i.e. dVIN- and/or LS-like lesions, were identified in the adjacent skin in 14 tumors (4.3%). Figure 1 shows the study algorithm. Table 1 shows the associated lesions identified in the skin for each specific HPV type in the whole series of 326 cases.

The HPV mRNA results were available in 304 out of the 326 tumors included in the series. Overall, 281/304 VSCC in which all the tests (HPV DNA,
HPV mRNA and p16 results) were available, were considered as conclusively associated with high-risk HPV. Among the VSCC conclusively associated with HPV, 261 (92.9%) were positive for both p16 and HPV mRNA, 11 (3.9%) were positive only for p16, and 9 (3.2%) were positive only for HPV mRNA. Twenty-four tumors were considered as non-conclusively associated with HPV due to negative p16 and HPV mRNA results. One hundred and eighty-seven of the 281 (66.5%) tumors conclusively associated with HPV showed HSIL/uVIN in the adjacent skin, whereas only 3/24 (12.5%) of the tumors with a non-conclusive association had adjacent HSIL/uVIN lesions (p<0.001). The skin precursor was also positive for p16 in all the p16 positive tumors showing HSIL/uVIN.

Among the 21 tumors with a low-risk or undetermined HPV type identified, one with a low-risk HPV (HPV44) had adjacent HSIL/uVIN, with both the invasive tumor and the HSIL/uVIN being negative for p16. Two tumors with an HPV of undetermined type showed adjacent HSIL/uVIN and were positive for p16.

**Unusual intraepithelial lesions**

Fourteen samples showed unusual intraepithelial lesion adjacent to the DNA HPV-positive VSCC. Seven cases (7/326; 2.1%) showed dVIN-like features, 5 cases (1.5%) showed adjacent LS-like lesions, and in 2 cases (0.6%) dVIN- and LS-like lesions were identified simultaneously. Finally, in one case (0.3%) LS-like features were identified in combination with typical HSIL/uVIN. Table 2 shows the age, the results of the p16 immunohistochemistry study of the tumor and the precursor lesion and HPV mRNA, the other
associated skin lesions and the histological sub-typing of the invasive tumor of all the cases with dVIN-like, LS-like or both lesions.

Six cases were conclusively associated with HPV (3 dVIN-like lesions, 2 LS-like lesions, 1 with combined dVIN/LS-like lesions). All were associated with HPV16 and were positive for both p16 and HPV mRNA. In all cases, the p16 was also positive in the adjacent dVIN-like lesion. Similarly, in the three cases with LS-like lesions, the skin overlying the LS-like area showed block positivity for p16, at least focally.

Another group of eight cases consisted of tumors with non-conclusive association with HPV and tumors caused by low-risk or undetermined HPV. All were positive for HPV DNA but were negative for p16 both in the tumor and in the precursor lesions. In all these cases, the HPV mRNA was either negative or not tested.

**dVIN-like and LS-like lesions in tumors conclusively associated with HPV**

In all dVIN-like cases identified in tumors conclusively associated with HPV, atypical keratinocytes were identified in a fully differentiated epithelium, with atypia strictly limited to the basal and parabasal layers, and with cells having abundant eosinophilic cytoplasm (Figure 2 A and 2B). The epithelium was atrophic or acanthotic with superficial parakeratosis. The rete ridges were elongated and branched. The keratinocytes in the epidermis were enlarged, had prominent desmosomes, abundant eosinophilic cytoplasm, large vesicular nuclei and macronucleoli. No extension to skin appendages was identified. None of these cases had the typical whole epithelial thickness architectural disarray, prominent cytologic atypia and nuclear pleomorphism typical of
HSIL/uVIN. A moderate lymphocytic infiltrate was present in the papillary dermis. In all cases, p16 showed diffuse block staining in the dVIN-like lesion with nuclear and cytoplasmic positivity involving at least the basal and suprabasal layers (Figure 2A’ and 2B’).

LS-like changes were characterized by broad condensation and hyalinization of the dermal collagen in the papillary dermis (Figure 3A). The epidermis showed slight to moderate hyperkeratosis. In contrast with typical LS lesions the rete ridges appeared elongated. In the two cases, p16 showed diffuse block staining in the epithelium overlying the hyalinized dermal collagen (Figure 3A’). A case showed combined LS-like (hyalinization of the dermal collagen in the papillary dermis) and dVIN-like lesions (atypia limited to the basal and parabasal layers in a fully differentiated epithelium with elongated rete ridges) (Figure 3B). The epithelium showed block positivity for p16.

**DISCUSSION**

In this study, we confirm that a subset of VSCC conclusively associated with HPV arises on intraepithelial lesions that have dVIN-like morphological features. In a subset of these cases, the association with HPV was conclusively established by positive HPV DNA result as well as the detection of HPV mRNA and a positive p16 result. Interestingly, in all these lesions p16 immunohistochemistry showed “block positivity”, both in the invasive tumor and in the intraepithelial lesion, a characteristic finding in HSIL/uVIN lesions. These findings indicate that HSIL/uVIN could have a wider morphological spectrum than traditionally accepted, with some lesions having a dVIN-like morphology. The proportion of these cases was very low, as this morphology was identified in only four out of 281 tumors conclusively associated with HPV (1.4%).
However, this distinction may be clinically relevant as vulvar HSIL/uVIN has a low progression potential and may regress spontaneously $^{15-17}$, whereas dVIN has shown a high potential for malignant transformation $^{8,18}$. Moreover, the identification of dVIN-like areas at the periphery in a VSCC may be considered as indirect evidence of an HPV-independent vulvar cancer $^{19}$. Interestingly, most of the women with VCSS conclusively associated with HPV with dVIN-like lesions were relatively old, a clinical characteristic typical of HPV-independent lesions.

This unusual pattern of HSIL/uVIN has not specifically been described previously. Nevertheless, a thorough review of the literature identified two possible additional cases. Yang et al $^{20}$ analyzed 12 cases of dVIN by PCR and/or ISH for various HPV types, and identified one case positive for HPV type 31/35/51 by *in situ* hybridization. The lesion was negative for p53 immunohistochemistry and the patient had multifocal intraepithelial lesions, some of which showed histological features overlapping with HSIL/uVIN.

Similarly, Haefner et al. identified HPV in 1 out of 7 cases of dVIN tested by PCR and *in situ* hybridization $^{21}$. Finally, a recent meta-analysis has estimated that the pooled prevalence of HPV-related dVIN-like lesions is about 2% $^9$.

The existence of HSIL/uVIN lesions with dVIN-like histological features is not surprising, because morphology studies have shown clear limitations in classifying vulvar tumors as HPV-associated or -independent $^3-6$. Additionally, we have previously described that a small percentage of HPV-independent VSCC have adjacent intraepithelial lesions with histological features mimicking basaloid type HSIL/uVIN $^4,7$. Finally, a number of studies have reported occasional lesions with overlapping morphological features of HSIL/uVIN and
dVIN; although most include either HPV-independent tumors or HPV detection was not made or was not reliable \(^\text{8,20,22}\).

In our study we found a strong correlation between the presence of high-risk viral type and adjacent HSIL/uVIN, which was present in 66.8% of the tumors. These results are in keeping with previous studies describing a strong association between high-risk viral type HSIL/uVIN and VSCC \(^\text{4,19,23}\). The percentage of HSIL/uVIN was similar for all tumors conclusively associated with HPV independently of the high-risk HPV type (and also with multiple infections) and ranged between 40-70%. In contrast, the percentage of HSIL/uVIN in tumors in which low-risk or undetermined HPV were detected was very low.

The low percentage of tumors non-conclusively associated with HPV (positive for HPV DNA, but negative for both p16 and HPV mRNA) that were associated with HSIL/uVIN was of note in our study. These findings suggest that many of these cases represent false positives for HPV DNA and are in keeping with the previously proposed criteria that HPV DNA alone should not be considered as sufficient evidence of HPV-association, unless a second marker (p16 or HPV mRNA) is also detected \(^\text{5,10}\). The almost constant positivity for p16 both in the invasive tumor and the intraepithelial lesion indicates that this marker can confidently be used as an HPV surrogate \(^\text{4,5,10}\).

The presence of LS-like changes in 3 tumors conclusively associated with HPV (2 cases with LS-like lesions and 1 with coincident dVIN/LS-like lesions) is striking. Although this coincidence could be largely circumstantial, and a woman with LS can be infected with HPV and also develop HSIL/uVIN, and subsequently, basaloid or warty VSCC, the block positivity for p16 in the skin overlying the LS-like lesions in all 3 tumors, even in skin with no atypical
features, raises the question of the presence of LS features in some HPV-associated intraepithelial lesions or the possible implication of HPV in the development of LS. A few studies have reported the presence of HPV in the epithelium with LS changes\(^{24}\) in HPV-associated VSCC, and it seems that these cases have fewer mutations than conventional HPV-negative LS\(^{25}\).

The pathogenesis of the VSCC in which low risk (HPV44) or undetermined HPV was detected type is uncertain. Although HPV44 is infrequent and considered to be of a low-risk viral type, it has occasionally been linked to the development of anogenital carcinomas through different mechanisms, without p16 overexpression\(^{26}\). However, the role of this HPV type is not well understood. To date, only one case of vulvar carcinoma of warty/basaloid morphology, in which HPV44 was detected, has been reported\(^ {26}\).

The main limitation of the present series is that HPV DNA or mRNA was only analyzed in the whole paraffin block containing both the precursor and the invasive tumor. Consequently, the possibility of a dVIN lesion, not related to the tumor, growing in the vicinity of a VSCC conclusively associated with HPV cannot be completely ruled out. However, the diffuse “block staining” for p16 identified in the dVIN-like lesions indicates that this hypothesis is rather unlikely.

A second limitation is that we did not study p53, which is frequently positive in dVIN\(^ {20}\). Nevertheless, in most of our cases a clear association with HPV16 was demonstrated and the dVIN-like lesions showed clear staining for p16, an unusual feature in HPV-negative dVIN\(^ {27}\).

In conclusion, in this large series of HPV-positive VSCCs with adjacent skin we found that a small proportion of tumors conclusively associated with HPV may arise in intraepithelial lesions that closely simulate dVIN and LS, the
intraepithelial precursors of HPV-independent VSCC. Awareness of this unusual feature may help to correctly classify these lesions as HPV-associated. In addition, p16 staining is an excellent surrogate marker of HPV infection and may help to identify these infrequent lesions.
FIGURE LEGENDS

**Figure 1.** Study algorithm

**Figure 2.** Differentiated vulvar intraepithelial neoplasia-like (dVIN-like) changes in two cases conclusively associated with human papillomavirus (HPV) type 16. Atypical keratinocytes are identified in a fully differentiated epithelium, with atypia limited to the basal and parabasal layers. The rete ridges are elongated and branched. The keratinocytes in the epidermis have abundant eosinophilic cytoplasm, and vesicular nuclei with macronucleoli. The whole epithelial thickness architectural disarray, prominent cytologic atypia and nuclear pleomorphism typical of high-grade squamous intraepithelial lesion (HSIL/uVIN) are absent. p16 immunohistochemistry shows block positive staining (A, A’ case number 1; B and B’ case number 3) (A and B, hematoxylin and eosin stain; A’ and B’ p16 immunohistochemical stain).

**Figure 3.** (A) Lichen sclerosus (LS)-like features in case number 5. There is clear condensation and hyalinization of the dermal collagen in the papillary dermis and subjacent inflammatory infiltrate. No atypical keratinocytes are identified. p16 shows block staining involving the whole thickness of the epithelium. Human papillomavirus (HPV) type 16 was identified (A’). (B) Case number 4 showing combined dVIN/LS-like lesions (case positive for HPV16). Hyalinization of the dermal collagen in the papillary dermis with subjacent inflammatory infiltrate coexists with elongation of the rete ridges and slight atypia of the basal layer. p16 immunohistochemistry shows block positive staining (B’).


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