"Histological characteristics of HPV-associated and -independent squamous cell carcinomas of the vulva: a study of 1594 cases"

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ABSTRACT

There are at least two different etio-pathogenic pathways for the development of vulvar squamous cell carcinoma (VSCC): one associated with infection by human papillomavirus (HPV) and another independent of HPV. We aimed to describe the histological characteristics of HPV-associated and HPV-independent tumors and to determine the best strategy to identify HPV in VSCC. A single paraffin block was available for review from a series of 1594 VSCCs. In all cases HPV DNA detection was analyzed using the SPF10PCR/DEIA/LiPA25 system and p16 immunohistochemistry (IHC). A tumor was considered as unquestionably HPV-associated if both HPV DNA and p16 IHC were positive. A tumor was considered indisputably HPV-independent if both HPV DNA and p16 IHC were negative. Two groups of tumors were classified as non-conclusive: 1) HPV DNA+/p16-; and 2) HPV DNA-/p16+. WHO typing and a thorough histological evaluation were conducted in all cases.

441 tumors were HPV DNA+ with 367 cases (23.0%) being HPV DNA+/p16+. These HPV DNA+/p16+ tumors were more frequently basaloid or warty (49.8%), but 36.5% were of the keratinizing type. 1153 tumors were HPV DNA-, with 1060 cases (66.5%) being HPV DNA-/p16-. These HPV DNA-/p16- tumors were mostly keratinizing (81.2%) but were occasionally basaloid or warty (5.2%). The features of HPV DNA-/p16+ cases (n=93) were similar to those of the HPV-associated VSCC, and HPV DNA+/p16- (n=74) cases had a more diverse profile, although they were more similar to HPV-independent tumors. Several histological characteristics were more frequently associated with HPV-related VSCC (koilocytotic-like change, necrosis, moderate to marked pleomorphism, invasive front in nests; $p<0.001$), however, none of these

characteristics allowed differentiation between HPV-associated and –independent VSCC. In conclusion, histological criteria do not allow differentiation between HPV-associated and –independent VSCC. p16 alone is a clinically easy strategy to determine HPV status in VSCC.

INTRODUCTION

Vulvar squamous cell carcinoma (VSCC) accounts for less than 5% of the gynecological malignancies but represents more than 90% of the malignant tumors of the vulva [1]. VSCCs can be subdivided into human papilloma virus (HPV)-associated and HPV-independent tumors, with HPV-associated tumors representing one-fifth to one-half of all VSCC [2–5] . Increasing evidence indicates that these two different types of VSCC not only have different epidemiological, clinical, pathological and molecular characteristics, but also a different clinical behavior. These differences stress the need of considering HPV-associated and HPV-independent vulvar tumors as two separate entities [6–8].

Most pathologists use clinico-pathological criteria to classify vulvar tumors as HPV-associated or –independent. The current WHO classification [9] recognizes several histological variants of VSCC, including the basaloid, warty, keratinizing and non-keratinizing subtypes. HPV-associated tumors are considered to have a warty or basaloid histology [10–13], and affect younger women [14–19]. Contrarily, HPV-independent lesions generally have a keratinizing morphology and occur in elderly women [14,20–24]. However, a number of studies indicate that these pathological features have a limited usefulness to determine HPV status [14,20–22]. Moreover, a comprehensive study analyzing histological characteristics of HPV-positive and –negative carcinomas has yet to be conducted.

Recent studies have shown that HPV DNA alone may be insufficient to identify HPV-associated cancers outside the cervix [25–28]. In recent years very sensitive and specific mRNA RT-PCR assays have been developed [28], but

these tests are extremely technically complex, and consequently are not adequate for pathology laboratories in routine daily practice.

p16 immunohistochemistry (IHC) has shown to be a useful tool to classify VSCC into HPV-associated or –independent and is considered a good surrogate marker of HPV association in the vulva and in other anatomical sites in which HPV-associated and HPV-independent tumors are present [2,14,16,22,28–32]. However, many studies evaluating p16 IHC have included a limited number of cases and, consequently, the reliability of p16 positivity as a single marker of HPV–association is not known.

Recently, the combination of two markers has been proposed as a reliable tool to provide robust results in terms of HPV status [2,28]. In this strategy, tumors positive for HPV DNA and p16 would be indisputably HPV-associated, whereas tumors negative for the two biomarkers would be classified as unquestionably HPV-independent. However, it is not known whether the tumors with intermediate features (HPV DNA positive and p16 negative and HPV DNA negative and p16 positive) truly represent HPV-associated or –independent tumors.

In this study, we analyzed a large series of 1594 tumors conducting a thorough histological analysis and detection of HPV using HPV DNA, p16 IHC and HPV mRNA. The study had three main goals: 1) to describe the histological characteristics of indisputably HPV-associated (HPV DNA+/p16+) and HPV-independent tumors (HPV DNA-/p16-); 2) to determine the frequency and the features of the tumors with non-conclusive features (HPV DNA-/p16+ and HPV DNA+/p16-); and 3) to determine the best strategy to differentiate HPV-associated and –independent tumors.

MATERIAL AND METHODS

Study design and materials

We reviewed all the invasive vulvar carcinomas previously analyzed in a retrospective cross-sectional survey coordinated by the Catalan Institute of Oncology (ICO, Barcelona-Spain) in collaboration with DDL Diagnostic Laboratory (Rijswijk, The Netherlands) [2,33]. In the present analysis we included all the cases fulfilling the following inclusion criteria: 1) invasive squamous carcinoma identified in the block; and 2) adequate material for histological analysis, HPV detection and typing and p16 immunohistochemical stain. The case recruitment protocols have been reported previously. Of the 1709 invasive vulvar tumors included in the study, 47 were excluded because of non-squamous histological types (i.e. basocellular or adenocarcinomas), and 68 were excluded because no material was available for p16 staining. Thus, the study included1594 formalin-fixed paraffin-embedded (FFPE) vulva specimens collected from pathology archives from 38 countries from all continents (Mali, Mozambique,Nigeria, and Senegal in Africa; Argentina,Brazil, Chile, Colombia, Ecuador, Guatemala, Honduras, Mexico, Paraguay, Uruguay, the United States and Venezuela in the Americas; Bangladesh, India, Israel, South Korea, Kuwait, Lebanon, Philippines, Taiwan and Turkey in Asia; Austria, Belarus, Bosnia-Herzegovina, Czech Republic, France, Germany, Greece, Italy, Poland, Portugal, Spain and the United Kingdom in Europe; and Australia and New Zealand in Oceania). Five hundred seventy-five cases (36.1%) were small biopsies from vulvar tumors and 1019 (63.9%) cases came from large surgical excisions or vulvectomy specimens. The study was approved by local and ICO ethics committees.

Histological evaluation

A single FFPE histological block was available from each case for review. Pathology evaluation was blind to HPV results and included a histological subtyping following the WHO 2014 classification [9]. Basaloid tumors are characterized for being composed of small undifferentiated cells with little keratinization and koilocytosis. Warty subtype is defined by its condylomatous features, pleomorphism, striking koilocythic atypia and frequent keratinization. Non-keratinizing carcinoma is composed of sheets or nests of polygonal squamous cells and absence of keratin pearls. The keratinizing type is characterized by differentiated cells with keratin pearls and absence of koilocytosis. When mixed features were identified, the tumor was classified according to the main component, but other secondary components were also recorded.

Additionally, the following histological characteristics were reported in all tumors: growth pattern (exophytic or endophytic), percentage of tumor nests showing keratin pearls, percentage of cells showing koilocytotic-like change, individual keratinization, and differentiated features (large, eosinophilic cytoplasm), percentage of tumor showing necrosis, extent of the inflammatory infiltrate adjacent to the tumor, and severity of the pleomorphism (these latter characteristics were semi-quantitatively evaluated as absent, mild, moderate or severe). The invasive front of the tumors was classified as having a nested (large or small nests) and diffuse pattern. Nested invasion consisted of either large geographic nests with frequent central comedo-type necrosis or small nests with frequent central keratin pearls [34]. A diffuse (or infiltrative) front was defined by irregular cords or single atypical cells in a so-called spray pattern,

often with surrounding desmoplastic stroma. Pattern evaluations were performed at low-power (x4 to x10) magnification. Figure 1 shows the main histological features evaluated in all the tumors.

Immunohistochemical staining for p16 and p53

All tumors were stained with p16 monoclonal antibody using the CINtec Histology Kit (clone E6H4; Roche-Mtm-Laboratories, Heidelberg, Germany) [35]. Only cases showing positivity in > 25% of tumor cells with strong and diffuse block staining of the basal layer were considered as positive ($p16^{INK4a}$ upregulation) [28,36].

p53 immunohistochemistry was performed in the first 192 cases of the whole series of 1594 tumors. p53 was detected with the monoclonal anti-body DO-7 (Dako, Carpinteria, CA, USA). p53 was considered positive when more than 25% of the neoplastic cells showed nuclear staining.

Tissue preparation, nucleic-acid isolation and HPV DNA detection

DNA extraction was performed on whole sections of the formalin-fixed paraffin-embedded (FFPE) tissue from the surgical specimen or from pre-treatment biopsy. The samples were serially sectioned on a microtome. The first and last sections (3 µm) were stained with H&E for histological confirmation of the diagnosis. In-between sections were collected in RNAase-free reaction tubes for DNA isolation (sandwich cutting technique). Sectioning and sample preparation were carried out with the highest measures to avoid cross-contamination. Paraffin blocks lacking tissue were cut in-between the patient samples as controls to ensure the lack of contamination. Processing and pathology diagnosis were done by the reference laboratory.

DNA extraction and HPV DNA detection has been previously described [37]. Briefly, HPV DNA detection was done using SPF10 PCR, DEIA and the LiPA25 system (version 1, Laboratory Biomedical Products, Rijswijk, The Netherlands).

HPV mRNA detection

The mRNA extraction and mRNA detection from tissue ribbons were performed as previously described [28]. For each case, HPV type-specific E6*I mRNA RT-PCR assays were performed for the HPV type(s) previously determined 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. Cases with HPV mRNA positive (HPV mRNA+) and/or ubiquitin C mRNA+ signal were considered “RNA valid”. All “RNA invalid” samples [i.e. cases that were HPV mRNA negative (HPV mRNA-) and ubiquitin C mRNA-] were analyzed a second time, and according to the signals obtained, they were classified as “RNA valid” or “RNA invalid”.

HPV E6*I mRNA was analyzed in all the HPV DNA positive cases and in a subset of 18 randomly selected HPV DNA cases as a negative control. A total of 18 HPV DNA- cases were tested (10 HPV DNA-/p16- and 8 HPV DNA-/p16+ cases).

Classification of tumors as HPV-associated and HPV-independent. Tumors with non-conclusive features

A tumor was considered as unquestionably HPV-associated if both HPV DNA and p16 IHC were positive. A tumor was considered indisputably HPV-independent if both HPV DNA detection and p16IHC were negative. These cases were considered the gold standard for HPV-positivity and –negativity, respectively. Two groups of tumors were classified as non-conclusive for HPV association: 1) tumors testing negative for HPV DNA but with positive staining for p16 (HPV DNA-/p16+); and 2) tumors testing positive for HPV DNA, but with negative result for p16 (HPV DNA+/p16-).

Statistical analysis

The results were reported as means, absolute frequencies and percentages. Chi-square analysis or Fisher's exact test were used for comparisons between nominal variables. Data were analyzed with the program SPSS (version 15.0; SPSS, Inc, Chicago, IL). All statistical tests were two-sided, and a p value of .05 or less was considered statistically significant.

RESULTS

HPV DNA, p16, p53, and histological typing: HPV-associated, HPV-independent tumors and tumors with non-conclusive features

Four hundred forty-one tumors (27.7%) were positive for HPV DNA, and 1153 (72.3%) were negative. Table 1 shows the WHO classification of the HPV-positive and -negative tumors. HPV-positive tumors were more frequently basaloid or warty (198 out of 441; 44.9%), while 42.0% were of the keratinizing type. HPV-negative tumors were more frequently keratinizing (905 out of 1153; 78.5%), but were occasionally of basaloid or warty type (88 out of 1153; 7.6%).

Mixed features, with presence of warty or basaloid along with keratinizing areas were identified in 96/1594 tumors (6.0%). Mixed features were observed in 30/441 (6.8%) HPV-positive tumors (6/160 [3.1%] basaloid, 4/38 [10.5%] warty, 5/56 [8.9%] non-keratinizing, 15/185 [8.1%] keratinizing) and in 66/1153 (5.7%) HPV-negative tumors (7/55 [12.7%] basaloid, 9/33 [27.3%] warty, 12/141 [8.5%] non-keratinizing, 38/905 [4.2%] keratinizing).

Three hundred sixty-seven out of 441 HPV DNA positive tumors (83.2%) were positive for p16 IHC; on the other hand, one thousand sixty out of 1153 HPV DNA negative tumors (91.9%) were negative for p16 IHC. The percentage of tumors of basaloid or warty type slightly increased in HPV-associated tumors (49.8%), whereas the percentage of histologically keratinizing tumors slightly increased in HPV-independent tumors (81.2%). Figure 2 shows an example of unquestionably HPV-associated VSCC with keratinizing histology (HPV DNA+/p16+), and two indisputably HPV-independent VSCC (HPV DNA-/p16-) with basaloid and warty characteristics.

One hundred sixty-seven tumors (10.5%) showed a non-conclusive profile. Ninety-three tumors were HPV DNA-/p16+; and 74 tumors were HPV DNA+/p16-. Table 2 shows the WHO classification and age distribution of the indisputably HPV-associated tumors (HPV DNA+/p16+), the unquestionably HPV-independent tumors (HPV DNA-/p16- tumors) and the tumors with non-conclusive profiles. The tumors with a HPV DNA-/p16+ profile were similar to the indisputably HPV-associated tumors in terms of WHO typing ($p>0.05$). The HPV DNA+/p16- tumors had indeterminate features, although they were more similar to the unquestionably HPV-independent tumors (slightly higher

proportion of warty-basaloid tumors in the HPV DNA+/p16- group compared with HPV DNA-/p16- group; p<0.001).

There were marked differences between these two groups in terms of age (61.7 ± 16.7 in the indisputably HPV-associated tumors vs. 71.7 ± 13.4, in the unquestionably HPV-independent tumors; p<0.001). Interestingly, the mean age of the patients with tumors with a HPV DNA-/p16+ profile was similar to that of the women with indisputably HPV-associated tumors (p>0.05). Contrarily, the HPV DNA+/p16- cases were similar in age distribution to the HPV-independent tumors.

From the subset of 192 tumors stained for p53, a positive result was observed in 2 out of 40 (5%) unquestionably HPV-associated tumors (HPV DNA+/p16+ profile) and in 88 out of the 140 (62.9%) unquestionably HPV-independent tumors (HPV DNA-/p16- profile). In the group of HPV DNA-/p16+ tumors, p53 was negative in all six tumors tested (0/6; 0%), whereas in the group of HPV DNA+/p16- tumors, 4/6 (66.7%) were positive for p53.

Histological characteristics of unquestionable HPV-associated and –independent tumors

Table 3 shows the histological characteristics of the indisputably HPV-associated tumors and the unquestionably HPV-independent tumors, as well as the characteristics of the HPV DNA positive and negative tumors without p16 IHC. Several histological characteristics were more frequently associated with HPV-associated tumors (koilocytotic-like change, necrosis, moderate to marked pleomorphism, invasive front in nests; p<0.001), whereas individual keratinization, keratin pearls, highly differentiated cells and a diffuse invasive

front were more frequently associated with HPV-independent VSCC ($p<0.001$). However, none of the histological features allowed reliable differentiation between the two types of VSCC as all of the characteristics were present in a proportion of both types of tumors.

HPV typing and histological classification

Table 4 shows the results of the HPV typing and the histological classification of the tumors according to the typing in the 441 cases positive for HPV DNA and in the group of 367 unquestionable HPV-associated tumors. HPV16 was, by far, the type most frequently identified.

p16 IHC was positive in over 90% of cases positive for HPV16 and 45, in over 80% of the cases positive for HPV18 and 33, in over 70% of multiple HPV infections and infections by other high-risk HPV, in 30% of infections by undetermined HPV types and was negative in tumors caused by low-risk HPV. The percentage of tumors with basaloid or warty histology ranged from 70% in HPV33 positive tumors to less than 40% in HPV 18 or HPV45 positive tumors.

HPV mRNA detection

Adequate material for mRNA detection was available in 402 out of the 441 (91.1%) HPV DNA positive tumors. HPV mRNA was identified in 350/402 tumors (87.1%). Five cases were considered as “RNA invalid”. Type-specific HPV mRNA was not analyzed in the tumors caused by undetermined (n=19), low-risk HPV (n=12), and in three tumors associated with high-risk types 30, 69, and 102, as the probes were not designed against these viruses. HPV mRNA was positive in 334/352 (94.9%) of the unquestionably HPV-associated tumors

(HPV DNA+/p16+) and in only 16/50 (32%) of the HPV DNA+/p16- tumors ($p<0.001$).

In the subset of 18 HPV DNA negative tumors tested for mRNA, eight tumors were HPV DNA-/p16+ and 10 HPV DNA-/p16-. Two out of eight (25%) HPV DNA-/p16+ tumors were positive for HPV mRNA, whereas none of the 10 unquestionably HPV-independent tumors (HPV DNA-/p16-) were positive for HPV mRNA.

DISCUSSION

The present study, which includes a large number of VSCCs, confirms previous results obtained by our group [14,16], showing that there is significant overlap between the histological types of VSCC and the association with HPV. Indeed, 36.5% of the unquestionable HPV-associated VSCCs were of the conventional keratinizing type, the histological type considered as characteristic of the HPV-independent pathway of VSCC [3,5,14,16,38–40] On the other hand, a small, albeit significant, number of indisputably HPV-independent VSCCs showed basaloid or warty features (5.2%), the histological types usually considered as associated with HPV-driven VSCCs [3,5,14,16,38,41,42]. This high prevalence of HPV infection among keratinizing VSCCs has also been observed by other authors, who have found percentages similar to or higher than those observed in our study [2,14,20,43–45]. Thus, the present results confirm the limited reliability of the current WHO classification of VSCCs in terms of HPV attribution [5,14,41].

The poor correlation observed between histological typing and HPV infection may be related, in part, by a certain degree of subjectivity in

subclassifying the tumors observed in our study as previously referred by some authors [14,41]. These difficulties are particularly challenging in the diagnosis of non-keratinizing carcinoma and to a lesser extent, warty carcinomas [14]. In addition, the presence of keratinization in basaloid carcinomas can hinder the differential diagnosis with poorly differentiated keratinizing squamous cell carcinomas. Moreover, mixed basaloid-keratinizing squamous carcinomas posing serious diagnostic problems have been described [17].

The marked overlap observed in all the histological features evaluated in our study clearly highlights the absence of specific criteria that allow classifying a tumor as HPV-associated or –independent. Indeed, although many of the histological findings evaluated in our study showed significant differences between the two groups, all the characteristics were found in a large proportion of HPV-associated and –independent tumors. A particularly unspecific feature is the presence of koilocytotic-like changes, which were observed in 11.6% of the HPV-independent VSCCs. Moreover, a marked interobserver variability in the separation between pseudokoilocytotic and true koilocytotic changes has been reported [14,46]. These findings further stress the poor validity of morphology alone to distinguish the two etio-pathogenic types of VSCC [5,14,20–22,25,41,47].

Our study shows that 10.5% of the tumors had non-conclusive features in terms of HPV attribution assessed by HPV DNA and p16. The most frequent group (HPV DNA-/p16+) was comparable to the unquestionably HPV-associated group in terms of age and histological typing. These findings indicate that these cases may represent false negative results of HPV DNA detection. In these cases the poor tissue quality could have contributed to the false negative HPV DNA result [2]. In contrast, the other non-conclusive group (HPV DNA+/p16-

) is more heterogeneous. It includes the unusual VSCCs (12 cases) harboring only a low-risk HPV, which have been shown to be negative for p16 IHC [48]. Some of the cases represented p16 negativity in truly HPV-positive tumors, as shown by the identification of 16 tumors positive not only for HPV DNA but also for HPV mRNA. However, it is likely that some of these cases are false positive HPV DNA cases. This group was more similar to the indisputably HPV-independent group in terms of histological characteristics and age range. Moreover, the infrequent presence of mRNA in these tumors (32.0% vs. 94.9% in the HPV DNA+/p16+ group) after excluding all cases not tested for mRNA HPV, as the test was not designed for low-risk and undetermined HPV types, is also in keeping with this hypothesis. As the whole FFPE block was tested for HPV detection without performing tumor microdissection, we cannot exclude the possibility of contamination from the adjacent embedded tissue not related to the tumor. Thus, our results suggest that HPV DNA detection alone could misclassify up to 10% of cases, whereas despite not being perfect, p16 immunohistochemistry could be a more reliable tool, offering even better sensitivity and specificity.

p53 IHC was positive in only 5% of the unquestionably HPV-associated tumors and in 66.7% of the unquestionably HPV-independent tumors. These results are in keeping with previous reports showing that p53 abnormalities are infrequent in HPV-associated tumors and present in about two thirds of HPV-independent tumors [14,49]. Interestingly, the percentage of p53 positivity in HPV DNA-/p16+ tumors was comparable to the unquestionably HPV-associated neoplasms whereas HPV DNA+/p16- tumors were more similar to the indisputably HPV-independent carcinomas. These results add further evidence

indicating that DNA-/p16+ tumors probably represent false negative results of HPV DNA detection, whereas many of the HPV DNA+/p16- tumors are false positive HPV DNA cases.

In situ hybridization assays that can detect HPV E6/E7 mRNA in FFPE tissue and allow visualization of the transcripts within tumor cells have recently become available. Although the value of these assays needs to be confirmed, they might help in the identification of HR-HPV-associated neoplasia in the vulva, the anus and the head and neck region [50].

The main strength of the present study is that it includes a very high number of VSCC, and in all the cases, a detailed histological review and thorough molecular analysis including HPV DNA, mRNA and p16 were performed. The correlation of all the data allowed the study to obtain more accurate evidence of HPV involvement in the large series of tumors [28].

The study also has some limitations. Only one FFPE block per case was available for review. This may, to some extent, have influenced the histological type distribution and, particularly, may have resulted in a relatively high percentage of non-keratinizing tumors, as keratin pearl formation may have been focal or absent in the area sampled. Additionally, although HPV DNA detection in this study has fulfilled strict quality controls, the different tissue processing techniques performed in a large variety of contributing laboratories could have had an impact on our results. However, the high number of cases analyzed and the strict quality controls used for p16 staining and HPV DNA and RNA detection have probably minimized this effect.

Finally, the complete absence of clinical and follow-up data prevents obtaining any conclusions on the prognostic implications of HPV status. Although there are some discordant results on the prognostic significance of HPV in VSCC [8,20], recent evidence indicates a strong association between positive p16 IHC and lower FIGO stage and negative lymph node metastasis [7], and that the presence of HPV DNA or positive p16 IHC is an independent prognostic factor [6].

In conclusion, the present study performed in a large number of cases of vulvar carcinomas confirms that histological criteria do not allow differentiation between HPV-associated and –independent VSCC. Although not perfect, p16 is a good surrogate marker that could even outperform the HPV DNA detection technique.

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LEGEND OF FIGURES

FIGURE 1. Histological characteristics evaluated in the study: **A)** koilocytotic-like change; **B)** necrosis; **C)** moderate to marked pleomorphism; **D)** individual keratinization, keratin pearls, and highly differentiated cells; **E)** invasive front in nests; **F)** diffuse invasive front. Hematoxylin and eosin staining.

FIGURE 2. A) Keratinizing VSCC unquestionably HPV-associated tumor (HPV DNA+/p16+); **B)** indisputably HPV-independent VSCC (HPV DNA-/p16-) of basaloid type; **C)** indisputably HPV-independent VSCC (HPV DNA-/p16-) of warty type. Hematoxylin and eosin staining (A, B, C) and p16 immunohistochemistry (A', B', C').

Table 1. WHO classification of the human papillomavirus (HPV)-associated and –independent tumors, defined by HPV DNA detection.

	HPV DNA+ (n=441)	**HPV DNA- (n=1153)**	
	n (%)	**n (%)**	**p-value**
Keratinizing	185 (42.0)	905 (78.5)	**0.000**
Non-keratinizing	56 (12.7)	141 (12.2)	0.786
Basaloid	160 (36.3)	55 (4.8)	**0.000**
Warty	38 (8.6)	33 (2.9)	**0.000**
Verrucous	1 (0.2)	15 (1.3)	0.087
Spindle cell	1 (0.2)	4 (0.3)	0.999

The statistical significant p-values are highlighted in bold (corrected p-value for multiple comparison; 0.05/6=0.008).

Table 2. WHO classification of the human papillomavirus (HPV)-associated and –independent tumors, defined by HPV DNA and p16 detection.

	Gold standard groups		Non-conclusive groups		
	HPV DNA+ p16+ (n=367)	**HPV DNA- p16- (n=1060)**	**HPV DNA- p16+ (n=93)**	**HPV DNA + p16- (n=74)**	
	n (%)	**n (%)**	**n (%)**	**n (%)**	**p-value[1]**
Keratinizing	134 (36.5)	861 (81.2)	44 (47.3)	51 (68.9)	**0.000**
Non-keratinizing	49 (13.4)	124 (11.7)	17 (18.3)	7 (9.5)	0.403
Basaloid	152 (41.4)	27 (2.5)	28 (30.1)	8 (10.8)	**0.000**
Warty	31 (8.4)	29 (2.7)	4 (4.3)	7 (9.5)	**0.000**
Verrucous	0 (0.0)	15 (1.4)	0 (0.0)	1 (1.4)	0.016
Spindle cell	1 (0.3)	4 (0.4)	0 (0.0)	0 (0.0)	1.000
Age range [min-max]	[22-97]	[18-104]	[24-94]	[33-92]	
Mean age (SD)	61.7 *(16.7)*	71.7 *(13.4)*	64.8 *(15.0)*	68.8 *(14.4)*	**0.000**

1) p-value: comparison of the gold standard for positivity and negativity (HPV DNA+ andp16+ vs HPV DNA- and p16-)
SD: Standard deviation.
The statistical significant p-values are highlighted in bold (corrected p-value for multiple comparisons; 0.05/6=0.008).

Table 3. Histological characteristics of the human papillomavirus (HPV)-associated and –independent tumors.

	HPV DNA+ (n=441) n (%)	HPV DNA- (n=1153) n (%)	HPV DNA+ and p16+ (n=367) n (%)	HPV DNA- and p16- (n=1060) n (%)	p-value[1]
Pattern of growth					
Exophytic	106 (24.0)	221 (19.2)	83 (22.6)	206 (19.4)	0.191
Endophytic	335 (76.0)	932 (80.8)	284 (77.4)	854 (80.6)	0.191
Koilocytotic-like change	154 (34.9)	145 (12.6)	121 (33.0)	123 (11.6)	**0.000**
Inflammatory infiltrate	283 (64.2)	720 (62.4)	234 (63.8)	658 (62.1)	0.566
Individual keratinization	223 (50.6)	832 (72.2)	170 (46.3)	780 (73.6)	**0.000**
Keratin pearls	307 (69.6)	1009 (87.5)	246 (67.0)	946 (89.2)	**0.000**
Differentiated cells ≥10%	339 (76.9)	1071 (92.9)	271 (73.8)	999 (94.2)	**0.000**
Necrosis	279 (63.3)	610 (52.9)	231 (62.9)	547 (51.6)	**0.000**
Pleomorphism moderate/marked	371 (84.1)	858 (74.4)	313 (85.3)	775 (73.1)	**0.000**
Invasive front					
Large or small nests	260 (59.0)	484 (42.0)	224 (61.0)	438 (41.3)	**0.000**
Diffuse	181 (41.0)	669 (58.0)	143 (39.0)	622 (58.7)	**0.000**

1) p-value: comparison of gold standards for positivity and negativity, that is HPV DNA+ AND p16+ vs HPV DNA- AND p16-
The statistical significant p-values are highlighted in bold (corrected p-value for multiple comparisons; 0.05/11=0.005).

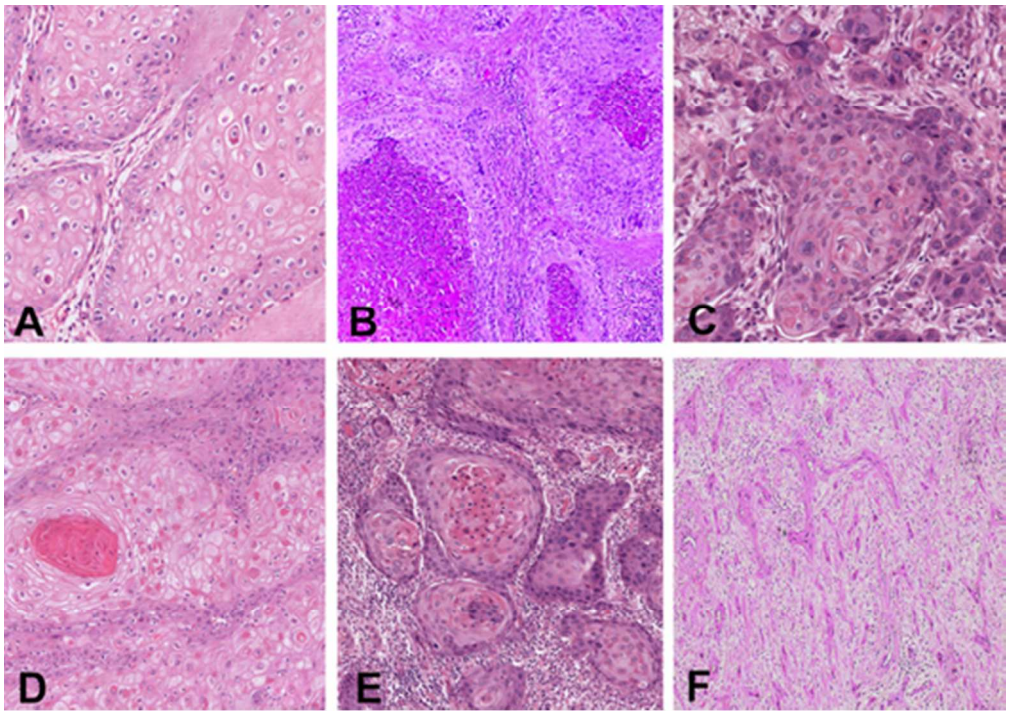
Table 4. Histological World Health Organization (WHO) classification according to human papillomavuirus (HPV) genotyping.

	HPV DNA positive (n=441)			HPV DNA positive and p16 positive (n=367)		
		WHO histological classification			WHO histological classification	
		Basaloid/warty	Keratinizing/non-keratinizing/verrucous/ spindle cell		Basaloid/warty	Keratinizing/non-keratinizing/verrucous/ spindle cell
HPV infection	**n**	**n (%)**	**n (%)**	**n**	**n (%)**	**n (%)**
Single HPV16	296	139 (47.0)	157 (53.0)	269	133 (49.4)	136 (50.6)
Single HPV33	23	15 (65.2)	8 (34.8)	20	14 (70.0)	6 (30.0)
Single HPV18	16	5 (31.3)	11 (68.7)	13	5 (38.5)	8 (61.5)
Single HPV45	13	5 (38.5)	8 (61.5)	13	5 (38.5)	8 (61.5)
Single high risk infections by HPV other than 16, 18, 33 or 45[a]	38	17(44.7)	21(55.3)	28	13(46.4)	15(53.6)
Multiple infections including at least one high risk HPV type[b]	24	11(45.8)	13 (54.2)	18	10 (55.5)	8(44.4)
Low-risk HPV infections[c]	12	2(16.7)	10(83.3)	0	-	-
Undetermined	19	4 (21.1)	15 (78.9)	6	4 (66.7)	2 (33.3)

[a] Includes infections by HPV 26 (1 case), 30 (1 case), 31 (4 cases), 35 (1 case), 39 (4 cases), 51 (2 cases), 52 (9 cases), 56 (6 cases), 58 (4 cases), 68 (2 cases), 69 (1 case), 70 (1 case), 73 (1 case), and 102 (1 case)

[b] Includes a) double infections by HPV6 and 16 (n=4); HPV16 and 18 (n=2); HPV16 and 31 (n=1); HPV16 and 33 (n=1); HPV16 and 51 (n=1); HPV18 and 11 (n=1); HPV18 and 44 (n=2); HPV18 and 74 (n=1); HPV31 and 33 (n=1); HPV31 and 42 (n=1); HPV33 and 56 (n=1); HPV35 and 66 (n=1); HPV44 and 45 (n=1); HPV44 and 58 (n=1); HPV44 and 66 (n=1); b) triple infections HPV31, 33 and 58 (n=1); HPV51, 68 and 73 (n=1); and c) quadruple infections HPV31, 33, 44 and 45 (n=1); and HPV51, 53, 54 and 58 (n=1)

[c] Includes single infections by HPV 6 (n=2), 11 (n=1), 44 (n=3), 53 (n=1), 61 (n=2), 74 (n=2) and a double infection 42 and 7 (n=1).



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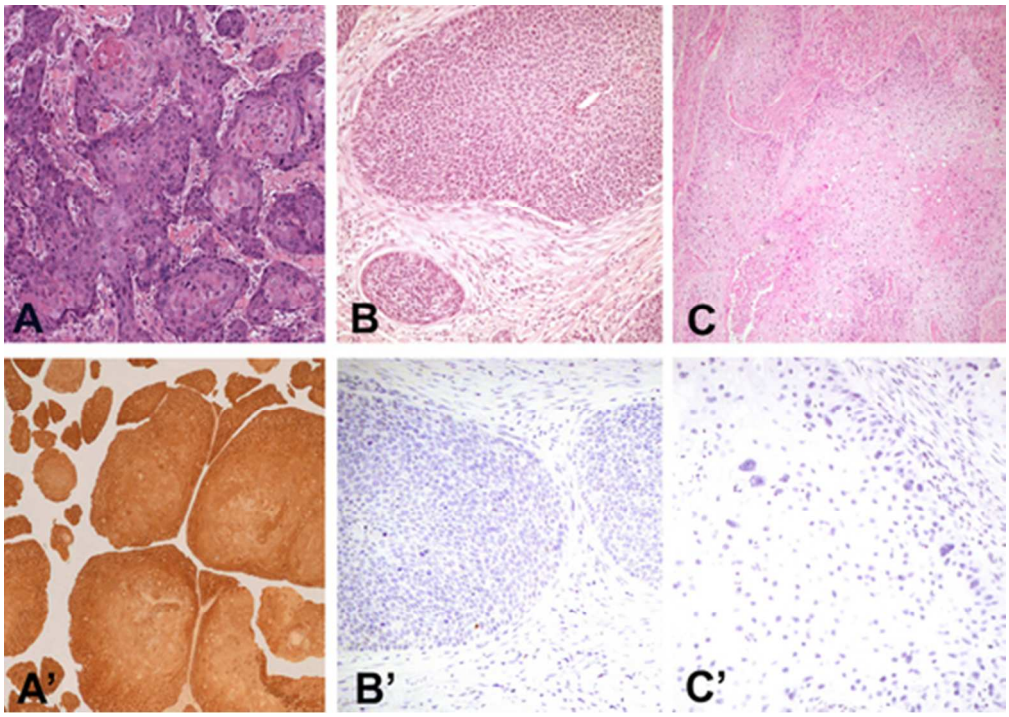

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