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1   **"Histological characteristics of HPV-associated and -independent**

2   **squamous cell carcinomas of the vulva: a study of 1594 cases"**

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23   HPV in vulvar cancer

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49   **ABSTRACT**

50   There are at least two different etio-pathogenic pathways for the development of

51   vulvar squamous cell carcinoma (VSCC): one associated with infection by

52   human papillomavirus (HPV) and another independent of HPV. We aimed to

53   describe the histological characteristics of HPV-associated and HPV-

54   independent tumors and to determine the best strategy to identify HPV in

55   VSCC. A single paraffin block was available for review from a series of 1594

56   VSCCs. In all cases HPV DNA detection was analyzed using the

57   SPF10PCR/DEIA/LiPA25 system and p16 immunohistochemistry (IHC). A tumor

58   was considered as unquestionably HPV-associated if both HPV DNA and p16

59   IHC were positive. A tumor was considered indisputably HPV-independent if

60   both HPV DNA and p16 IHC were negative. Two groups of tumors were

61   classified as non-conclusive: 1) HPV DNA+/p16-; and 2) HPV DNA-/p16+. WHO

62   typing and a thorough histological evaluation were conducted in all cases.

63   441 tumors were HPV DNA+ with 367 cases (23.0%) being HPV DNA+/p16+.

64   These HPV DNA+/p16+ tumors were more frequently basaloid or warty

65   (49.8%), but 36.5% were of the keratinizing type. 1153 tumors were HPV DNA-,

66   with 1060 cases (66.5%) being HPV DNA-/p16-. These HPV DNA-/p16- tumors

67   were mostly keratinizing (81.2%) but were occasionally basaloid or warty

68   (5.2%). The features of HPV DNA-/p16+ cases (n=93) were similar to those of

69   the HPV-associated VSCC, and HPV DNA+/p16- (n=74) cases had a more

70   diverse profile, although they were more similar to HPV-independent tumors.

71   Several histological characteristics were more frequently associated with HPV-

72   related VSCC (koilocytotic-like change, necrosis, moderate to marked

73   pleomorphism, invasive front in nests; p<0.001), however, none of these

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74   characteristics allowed differentiation between HPV-associated and –

75   independent VSCC. In conclusion, histological criteria do not allow

76   differentiation between HPV-associated and –independent VSCC. p16 alone is

77   a clinically easy strategy to determine HPV status in VSCC.

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79   **INTRODUCTION**

80   Vulvar squamous cell carcinoma (VSCC) accounts for less than 5% of the

81   gynecological malignancies but represents more than 90% of the malignant

82   tumors of the vulva [1]. VSCCs can be subdivided into human papilloma virus

83   (HPV)-associated and HPV-independent tumors, with HPV-associated tumors

84   representing one-fifth to one-half of all VSCC [2–5]. Increasing evidence indicates

85   that these two different types of VSCC not only have different epidemiological,

86   clinical, pathological and molecular characteristics, but also a different clinical

87   behavior. These differences stress the need of considering HPV-associated

88   and HPV-independent vulvar tumors as two separate entities [6–8].

89   Most pathologists use clinico-pathological criteria to classify vulvar tumors

90   as HPV-associated or –independent. The current WHO classification [9]

91   recognizes several histological variants of VSCC, including the basaloid, warty,

92   keratinizing and non-keratinizing subtypes. HPV-associated tumors are

93   considered to have a warty or basaloid histology [10–13], and affect younger

94   women [14–19]. Contrarily, HPV-independent lesions generally have a keratinizing

95   morphology and occur in elderly women [14,20–24]. However, a number of studies

96   indicate that these pathological features have a limited usefulness to determine

97   HPV status [14,20–22]. Moreover, a comprehensive study analyzing histological

98   characteristics of HPV-positive and –negative carcinomas has yet to be

99   conducted.

100   Recent studies have shown that HPV DNA alone may be insufficient to

101   identify HPV-associated cancers outside the cervix [25–28]. In recent years very

102   sensitive and specific mRNA RT-PCR assays have been developed [28], but

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103   these tests are extremely technically complex, and consequently are not

104   adequate for pathology laboratories in routine daily practice.

105   p16 immunohistochemistry (IHC) has shown to be a useful tool to classify

106   VSCC into HPV-associated or –independent and is considered a good

107   surrogate marker of HPV association in the vulva and in other anatomical sites

108   in which HPV-associated and HPV-independent tumors are present [2,14,16,22,28–

109   32]. However, many studies evaluating p16 IHC have included a limited number

110   of cases and, consequently, the reliability of p16 positivity as a single marker of

111   HPV–association is not known.

112   Recently, the combination of two markers has been proposed as a reliable

113   tool to provide robust results in terms of HPV status [2,28]. In this strategy, tumors

114   positive for HPV DNA and p16 would be indisputably HPV-associated, whereas

115   tumors negative for the two biomarkers would be classified as unquestionably

116   HPV-independent. However, it is not known whether the tumors with

117   intermediate features (HPV DNA positive and p16 negative and HPV DNA

118   negative and p16 positive) truly represent HPV-associated or –independent

119   tumors.

120   In this study, we analyzed a large series of 1594 tumors conducting a

121   thorough histological analysis and detection of HPV using HPV DNA, p16 IHC

122   and HPV mRNA. The study had three main goals: 1) to describe the histological

123   characteristics of indisputably HPV-associated (HPV DNA+/p16+) and HPV-

124   independent tumors (HPV DNA-/p16-); 2) to determine the frequency and the

125   features of the tumors with non-conclusive features (HPV DNA-/p16+ and HPV

126   DNA+/p16-); and 3) to determine the best strategy to differentiate HPV-

127   associated and –independent tumors.

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128 **MATERIAL AND METHODS**

129 *Study design and materials*

130 We reviewed all the invasive vulvar carcinomas previously analyzed in a

131 retrospective cross-sectional survey coordinated by the Catalan Institute of

132 Oncology (ICO, Barcelona-Spain) in collaboration with DDL Diagnostic

133 Laboratory (Rijswijk, The Netherlands) [2,33]. In the present analysis we included

134 all the cases fulfilling the following inclusion criteria: 1) invasive squamous

135 carcinoma identified in the block; and 2) adequate material for histological

136 analysis, HPV detection and typing and p16 immunohistochemical stain. The

137 case recruitment protocols have been reported previously. Of the 1709 invasive

138 vulvar tumors included in the study, 47 were excluded because of non-

139 squamous histological types (i.e. basocellular or adenocarcinomas), and 68

140 were excluded because no material was available for p16 staining. Thus, the

141 study included1594 formalin-fixed paraffin-embedded (FFPE) vulva specimens

142 collected from pathology archives from 38 countries from all continents (Mali,

143 Mozambique,Nigeria, and Senegal in Africa; Argentina,Brazil, Chile, Colombia,

144 Ecuador, Guatemala, Honduras, Mexico, Paraguay, Uruguay, the United States

145 and Venezuela in the Americas; Bangladesh, India, Israel, South Korea, Kuwait,

146 Lebanon, Philippines, Taiwan and Turkey in Asia; Austria, Belarus, Bosnia-

147 Herzegovina, Czech Republic, France, Germany, Greece, Italy, Poland,

148 Portugal, Spain and the United Kingdom in Europe; and Australia and New

149 Zealand in Oceania). Five hundred seventy-five cases (36.1%) were small

150 biopsies from vulvar tumors and 1019 (63.9%) cases came from large surgical

151 excisions or vulvectomy specimens. The study was approved by local and ICO

152 ethics committees.

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153 *Histological evaluation*

154   A single FFPE histological block was available from each case for review.

155 Pathology evaluation was blind to HPV results and included a histological

156 subtyping following the WHO 2014 classification [9]. Basaloid tumors are

157 characterized for being composed of small undifferentiated cells with little

158 keratinization and koilocytosis. Warty subtype is defined by its condylomatous

159 features, pleomorphism, striking koilocythic atypia and frequent keratinization.

160 Non-keratinizing carcinoma is composed of sheets or nests of polygonal

161 squamous cells and absence of keratin pearls. The keratinizing type is

162 characterized by differentiated cells with keratin pearls and absence of

163 koilocytosis. When mixed features were identified, the tumor was classified

164 according to the main component, but other secondary components were also

165 recorded.

166   Additionally, the following histological characteristics were reported in all

167 tumors: growth pattern (exophytic or endophytic), percentage of tumor nests

168 showing keratin pearls, percentage of cells showing koilocytotic-like change,

169 individual keratinization, and differentiated features (large, eosinophilic

170 cytoplasm), percentage of tumor showing necrosis, extent of the inflammatory

171 infiltrate adjacent to the tumor, and severity of the pleomorphism (these latter

172 characteristics were semi-quantitatively evaluated as absent, mild, moderate or

173 severe). The invasive front of the tumors was classified as having a nested

174 (large or small nests) and diffuse pattern. Nested invasion consisted of either

175 large geographic nests with frequent central comedo-type necrosis or small

176 nests with frequent central keratin pearls [34]. A diffuse (or infiltrative) front was

177 defined by irregular cords or single atypical cells in a so-called spray pattern,

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178 often with surrounding desmoplastic stroma. Pattern evaluations were

179 performed at low-power (x4 to x10) magnification. Figure 1 shows the main

180 histological features evaluated in all the tumors.

181 *Immunohistochemical staining for p16 and p53*

182 All tumors were stained with p16 monoclonal antibody using the CINtec

183 Histology Kit (clone E6H4; Roche-Mtm-Laboratories, Heidelberg, Germany) [35].

184 Only cases showing positivity in > 25% of tumor cells with strong and diffuse

185 block staining of the basal layer were considered as positive (p16^{INK4a}

186 upregulation) [28,36].

187 p53 immunohistochemistry was performed in the first 192 cases of the

188 whole series of 1594 tumors. p53 was detected with the monoclonal anti-body

189 DO-7 (Dako, Carpinteria, CA, USA). p53 was considered positive when more

190 than 25% of the neoplastic cells showed nuclear staining.

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

192 DNA extraction was performed on whole sections of the formalin-fixed

193 paraffin-embedded (FFPE) tissue from the surgical specimen or from pre-

194 treatment biopsy. The samples were serially sectioned on a microtome. The first

195 and last sections (3 μm) were stained with H&E for histological confirmation of

196 the diagnosis. In-between sections were collected in RNAase-free reaction

197 tubes for DNA isolation (sandwich cutting technique). Sectioning and sample

198 preparation were carried out with the highest measures to avoid cross-

199 contamination. Paraffin blocks lacking tissue were cut in-between the patient

200 samples as controls to ensure the lack of contamination. Processing and

201 pathology diagnosis were done by the reference laboratory.

202   DNA extraction and HPV DNA detection has been previously described [37].

203   Briefly, HPV DNA detection was done using SPF10 PCR, DEIA and the LiPA25

204   system (version 1, Laboratory Biomedical Products, Rijswijk, The Netherlands).

205   *HPV mRNA detection*

206   The mRNA extraction and mRNA detection from tissue ribbons were

207   performed as previously described [28]. For each case, HPV type-specific E6*I

208   mRNA RT-PCR assays were performed for the HPV type(s) previously

209   determined by genotyping and for a cellular ubiquitin C gene as a control for

210   tissue quality. HPV E6*I mRNA assays were developed for the following HPV

211   types: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 70, 73,

212   and 82. A second assay was performed to assess the presence of HPV16 E6*I

213   mRNA in all cases, irrespective of the HPV DNA result. Cases with HPV mRNA

214   positive (HPV mRNA+) and/or ubiquitin C mRNA+ signal were considered "RNA

215   valid". All "RNA invalid" samples [i.e. cases that were HPV mRNA negative

216   (HPV mRNA-) and ubiquitin C mRNA-] were analyzed a second time, and

217   according to the signals obtained, they were classified as "RNA valid" or "RNA

218   invalid".

219   HPV E6*I mRNA was analyzed in all the HPV DNA positive cases and in a

220   subset of 18 randomly selected HPV DNA cases as a negative control. A total of

221   18 HPV DNA- cases were tested (10 HPV DNA-/p16- and 8 HPV DNA-/p16+

222   cases).

223

224   *Classification of tumors as HPV-associated and HPV-independent. Tumors with*

225   *non-conclusive features*

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226   A tumor was considered as unquestionably HPV-associated if both HPV

227   DNA and p16 IHC were positive. A tumor was considered indisputably HPV-

228   independent if both HPV DNA detection and p16IHC were negative. These

229   cases were considered the gold standard for HPV-positivity and –negativity,

230   respectively. Two groups of tumors were classified as non-conclusive for HPV

231   association: 1) tumors testing negative for HPV DNA but with positive staining

232   for p16 (HPV DNA-/p16+); and 2) tumors testing positive for HPV DNA, but with

233   negative result for p16 (HPV DNA+/p16-).

234   *Statistical analysis*

235   The results were reported as means, absolute frequencies and

236   percentages. Chi-square analysis or Fisher's exact test were used for

237   comparisons between nominal variables. Data were analyzed with the program

238   SPSS (version 15.0; SPSS, Inc, Chicago, IL). All statistical tests were two-

239   sided, and a p value of .05 or less was considered statistically significant.

240   **RESULTS**

241   *HPV DNA, p16, p53, and histological typing: HPV-associated, HPV-independent*

242   *tumors and tumors with non-conclusive features*

243   Four hundred forty-one tumors (27.7%) were positive for HPV DNA, and

244   1153 (72.3%) were negative. Table 1 shows the WHO classification of the HPV-

245   positive and -negative tumors. HPV-positive tumors were more frequently

246   basaloid or warty (198 out of 441; 44.9%), while 42.0% were of the keratinizing

247   type. HPV-negative tumors were more frequently keratinizing (905 out of 1153;

248   78.5%), but were occasionally of basaloid or warty type (88 out of 1153; 7.6%).

249   Mixed features, with presence of warty or basaloid along with keratinizing

250 areas were identified in 96/1594 tumors (6.0%). Mixed features were observed

251 in 30/441 (6.8%) HPV-positive tumors (6/160 [3.1%] basaloid, 4/38 [10.5%]

252 warty, 5/56 [8.9%] non-keratinizing, 15/185 [8.1%] keratinizing) and in 66/1153

253 (5.7%) HPV-negative tumors (7/55 [12.7%] basaloid, 9/33 [27.3%] warty, 12/141

254 [8.5%] non-keratinizing, 38/905 [4.2%] keratinizing).

255   Three hundred sixty-seven out of 441 HPV DNA positive tumors (83.2%)

256 were positive for p16 IHC; on the other hand, one thousand sixty out of 1153

257 HPV DNA negative tumors (91.9%) were negative for p16 IHC. The percentage

258 of tumors of basaloid or warty type slightly increased in HPV-associated tumors

259 (49.8%), whereas the percentage of histologically keratinizing tumors slightly

260 increased in HPV-independent tumors (81.2%). Figure 2 shows an example of

261 unquestionably HPV-associated VSCC with keratinizing histology (HPV

262 DNA+/p16+), and two indisputably HPV-independent VSCC (HPV DNA-/p16-)

263 with basaloid and warty characteristics.

264   One hundred sixty-seven tumors (10.5%) showed a non-conclusive profile.

265 Ninety-three tumors were HPV DNA-/p16+; and 74 tumors were HPV

266 DNA+/p16-. Table 2 shows the WHO classification and age distribution of the

267 indisputably HPV-associated tumors (HPV DNA+/p16+), the unquestionably

268 HPV-independent tumors (HPV DNA-/p16- tumors) and the tumors with non-

269 conclusive profiles. The tumors with a HPV DNA-/p16+ profile were similar to

270 the indisputably HPV-associated tumors in terms of WHO typing (p>0.05). The

271 HPV DNA+/p16- tumors had indeterminate features, although they were more

272 similar to the unquestionably HPV-independent tumors (slightly higher

273   proportion of warty-basaloid tumors in the HPV DNA+/p16- group compared

274   with HPV DNA-/p16- group; p<0.001).

275   There were marked differences between these two groups in terms of age

276   (61.7 ± 16.7 in the indisputably HPV-associated tumors vs. 71.7 ± 13.4, in the

277   unquestionably HPV-independent tumors; p<0.001). Interestingly, the mean age

278   of the patients with tumors with a HPV DNA-/p16+ profile was similar to that of

279   the women with indisputably HPV-associated tumors (p>0.05). Contrarily, the

280   HPV DNA+/p16- cases were similar in age distribution to the HPV-independent

281   tumors.

282   From the subset of 192 tumors stained for p53, a positive result was

283   observed in 2 out of 40 (5%) unquestionably HPV-associated tumors (HPV

284   DNA+/p16+ profile) and in 88 out of the 140 (62.9%) unquestionably HPV-

285   independent tumors (HPV DNA-/p16- profile). In the group of HPV DNA-/p16+

286   tumors, p53 was negative in all six tumors tested (0/6; 0%), whereas in the

287   group of HPV DNA+/p16- tumors, 4/6 (66.7%) were positive for p53.

288   *Histological characteristics of unquestionable HPV-associated and –*

289   *independent tumors*

290   Table 3 shows the histological characteristics of the indisputably HPV-

291   associated tumors and the unquestionably HPV-independent tumors, as well as

292   the characteristics of the HPV DNA positive and negative tumors without p16

293   IHC. Several histological characteristics were more frequently associated with

294   HPV-associated tumors (koilocytotic-like change, necrosis, moderate to marked

295   pleomorphism, invasive front in nests; p<0.001), whereas individual

296   keratinization, keratin pearls, highly differentiated cells and a diffuse invasive

297   front were more frequently associated with HPV-independent VSCC (p<0.001).

298   However, none of the histological features allowed reliable differentiation

299   between the two types of VSCC as all of the characteristics were present in a

300   proportion of both types of tumors.

301   *HPV typing and histological classification*

302   Table 4 shows the results of the HPV typing and the histological

303   classification of the tumors according to the typing in the 441 cases positive for

304   HPV DNA and in the group of 367 unquestionable HPV-associated tumors.

305   HPV16 was, by far, the type most frequently identified.

306   p16 IHC was positive in over 90% of cases positive for HPV16 and 45, in

307   over 80% of the cases positive for HPV18 and 33, in over 70% of multiple HPV

308   infections and infections by other high-risk HPV, in 30% of infections by

309   undetermined HPV types and was negative in tumors caused by low-risk HPV.

310   The percentage of tumors with basaloid or warty histology ranged from 70% in

311   HPV33 positive tumors to less than 40% in HPV 18 or HPV45 positive tumors.

312   *HPV mRNA detection*

313   Adequate material for mRNA detection was available in 402 out of the 441

314   (91.1%) HPV DNA positive tumors. HPV mRNA was identified in 350/402

315   tumors (87.1%). Five cases were considered as "RNA invalid". Type-specific

316   HPV mRNA was not analyzed in the tumors caused by undetermined (n=19),

317   low-risk HPV (n=12), and in three tumors associated with high-risk types 30, 69,

318   and 102, as the probes were not designed against these viruses. HPV mRNA

319   was positive in 334/352 (94.9%) of the unquestionably HPV-associated tumors

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320   (HPV DNA+/p16+) and in only 16/50 (32%) of the HPV DNA+/p16- tumors

321   (p<0.001).

322   In the subset of 18 HPV DNA negative tumors tested for mRNA, eight

323   tumors were HPV DNA-/p16+ and 10 HPV DNA-/p16-. Two out of eight (25%)

324   HPV DNA-/p16+ tumors were positive for HPV mRNA, whereas none of the 10

325   unquestionably HPV-independent tumors (HPV DNA-/p16-) were positive for

326   HPV mRNA.

327   **DISCUSSION**

328   The present study, which includes a large number of VSCCs, confirms

329   previous results obtained by our group [14,16], showing that there is significant

330   overlap between the histological types of VSCC and the association with HPV.

331   Indeed, 36.5% of the unquestionable HPV-associated VSCCs were of the

332   conventional keratinizing type, the histological type considered as characteristic

333   of the HPV-independent pathway of VSCC [3,5,14,16,38–40] On the other hand, a

334   small, albeit significant, number of indisputably HPV-independent VSCCs

335   showed basaloid or warty features (5.2%), the histological types usually

336   considered as associated with HPV-driven VSCCs [3,5,14,16,38,41,42]. This high

337   prevalence of HPV infection among keratinizing VSCCs has also been

338   observed by other authors, who have found percentages similar to or higher

339   than those observed in our study [2,14,20,43–45]. Thus, the present results confirm

340   the limited reliability of the current WHO classification of VSCCs in terms of

341   HPV attribution [5,14,41].

342   The poor correlation observed between histological typing and HPV

343   infection may be related, in part, by a certain degree of subjectivity in

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344 subclassifying the tumors observed in our study as previously referred by some

345 authors [14,41]. These difficulties are particularly challenging in the diagnosis of

346 non-keratinizing carcinoma and to a lesser extent, warty carcinomas [14]. In

347 addition, the presence of keratinization in basaloid carcinomas can hinder the

348 differential diagnosis with poorly differentiated keratinizing squamous cell

349 carcinomas. Moreover, mixed basaloid-keratinizing squamous carcinomas

350 posing serious diagnostic problems have been described [17].

351   The marked overlap observed in all the histological features evaluated in

352 our study clearly highlights the absence of specific criteria that allow classifying

353 a tumor as HPV-associated or –independent. Indeed, although many of the

354 histological findings evaluated in our study showed significant differences

355 between the two groups, all the characteristics were found in a large proportion

356 of HPV-associated and –independent tumors. A particularly unspecific feature is

357 the presence of koilocytotic-like changes, which were observed in 11.6% of the

358 HPV-independent VSCCs. Moreover, a marked interobserver variability in the

359 separation between pseudokoilocytotic and true koilocytotic changes has been

360 reported [14,46]. These findings further stress the poor validity of morphology

361 alone to distinguish the two etio-pathogenic types of VSCC [5,14,20–22,25,41,47].

362   Our study shows that 10.5% of the tumors had non-conclusive features in

363 terms of HPV attribution assessed by HPV DNA and p16. The most frequent

364 group (HPV DNA-/p16+) was comparable to the unquestionably HPV-

365 associated group in terms of age and histological typing. These findings indicate

366 that these cases may represent false negative results of HPV DNA detection. In

367 these cases the poor tissue quality could have contributed to the false negative

368 HPV DNA result [2]. In contrast, the other non-conclusive group (HPV DNA+/p16-

369   ) is more heterogeneous. It includes the unusual VSCCs (12 cases) harboring

370   only a low-risk HPV, which have been shown to be negative for p16 IHC [48].

371   Some of the cases represented p16 negativity in truly HPV-positive tumors, as

372   shown by the identification of 16 tumors positive not only for HPV DNA but also

373   for HPV mRNA. However, it is likely that some of these cases are false positive

374   HPV DNA cases. This group was more similar to the indisputably HPV-

375   independent group in terms of histological characteristics and age range.

376   Moreover, the infrequent presence of mRNA in these tumors (32.0% vs. 94.9%

377   in the HPV DNA+/p16+ group) after excluding all cases not tested for mRNA

378   HPV, as the test was not designed for low-risk and undetermined HPV types, is

379   also in keeping with this hypothesis. As the whole FFPE block was tested for

380   HPV detection without performing tumor microdissection, we cannot exclude the

381   possibility of contamination from the adjacent embedded tissue not related to

382   the tumor. Thus, our results suggest that HPV DNA detection alone could

383   misclassify up to 10% of cases, whereas despite not being perfect, p16

384   immunohistochemistry could be a more reliable tool, offering even better

385   sensitivity and specificity.

386   p53 IHC was positive in only 5% of the unquestionably HPV-associated

387   tumors and in 66.7% of the unquestionably HPV-independent tumors. These

388   results are in keeping with previous reports showing that p53 abnormalities are

389   infrequent in HPV-associated tumors and present in about two thirds of HPV-

390   independent tumors [14,49]. Interestingly, the percentage of p53 positivity in HPV

391   DNA-/p16+ tumors was comparable to the unquestionably HPV-associated

392   neoplasms whereas HPV DNA+/p16- tumors were more similar to the

393   indisputably HPV-independent carcinomas. These results add further evidence

394 indicating that DNA-/p16+ tumors probably represent false negative results of

395 HPV DNA detection, whereas many of the HPV DNA+/p16- tumors are false

396 positive HPV DNA cases.

397   *In situ* hybridization assays that can detect HPV E6/E7 mRNA in FFPE

398 tissue and allow visualization of the transcripts within tumor cells have recently

399 become available. Although the value of these assays needs to be confirmed,

400 they might help in the identification of HR-HPV-associated neoplasia in the

401 vulva, the anus and the head and neck region [50].

402   The main strength of the present study is that it includes a very high

403 number of VSCC, and in all the cases, a detailed histological review and

404 thorough molecular analysis including HPV DNA, mRNA and p16 were

405 performed. The correlation of all the data allowed the study to obtain more

406 accurate evidence of HPV involvement in the large series of tumors [28].

407   The study also has some limitations. Only one FFPE block per case was

408 available for review. This may, to some extent, have influenced the histological

409 type distribution and, particularly, may have resulted in a relatively high

410 percentage of non-keratinizing tumors, as keratin pearl formation may have

411 been focal or absent in the area sampled. Additionally, although HPV DNA

412 detection in this study has fulfilled strict quality controls, the different tissue

413 processing techniques performed in a large variety of contributing laboratories

414 could have had an impact on our results. However, the high number of cases

415 analyzed and the strict quality controls used for p16 staining and HPV DNA and

416 RNA detection have probably minimized this effect.

417   Finally, the complete absence of clinical and follow-up data prevents

418   obtaining any conclusions on the prognostic implications of HPV status.

419   Although there are some discordant results on the prognostic significance of

420   HPV in VSCC [8,20], recent evidence indicates a strong association between

421   positive p16 IHC and lower FIGO stage and negative lymph node metastasis [7],

422   and that the presence of HPV DNA or positive p16 IHC is an independent

423   prognostic factor [6].

424   In conclusion, the present study performed in a large number of cases of

425   vulvar carcinomas confirms that histological criteria do not allow differentiation

426   between HPV-associated and –independent VSCC. Although not perfect, p16 is

427   a good surrogate marker that could even outperform the HPV DNA detection

428   technique.

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663 **LEGEND OF FIGURES**

664 **FIGURE 1.** Histological characteristics evaluated in the study: **A)** koilocytotic-like

665 change; **B)** necrosis; **C)** moderate to marked pleomorphism; **D)** individual

666 keratinization, keratin pearls, and highly differentiated cells; **E)** invasive front in

667 nests; **F)** diffuse invasive front. Hematoxylin and eosin staining.

668 **FIGURE 2. A)** Keratinizing VSCC unquestionably HPV-associated tumor (HPV

669 DNA+/p16+); **B)** indisputably HPV-independent VSCC (HPV DNA-/p16-) of

670 basaloid type; **C)** indisputably HPV-independent VSCC (HPV DNA-/p16-) of

671 warty type. Hematoxylin and eosin staining (A, B, C) and p16

672 immunohistochemistry (A', B', C').

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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 infection	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
	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).
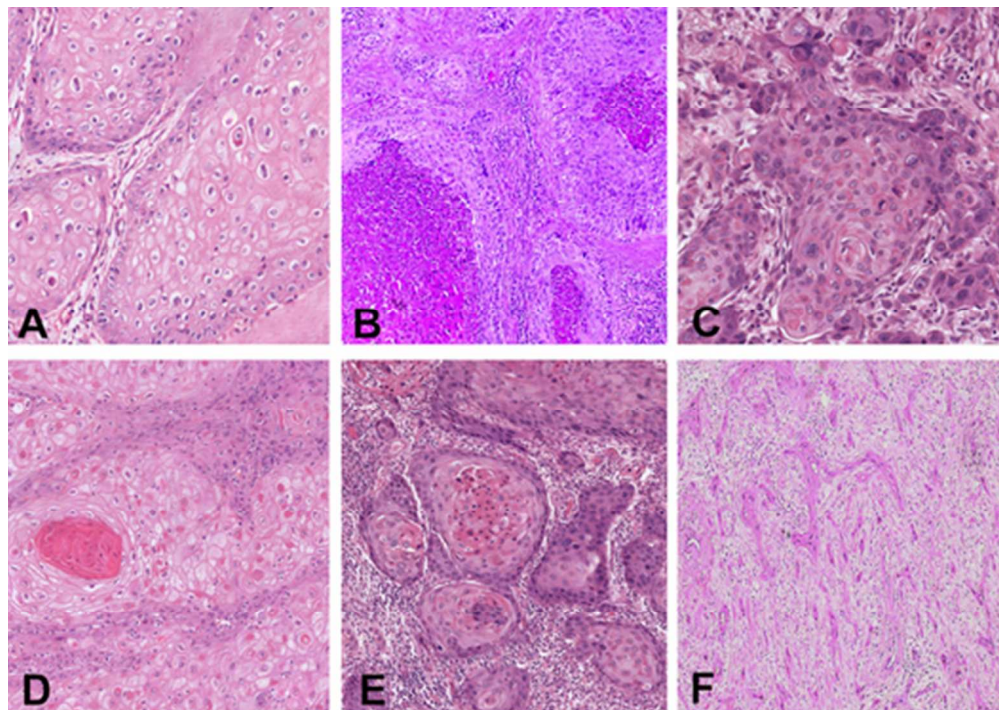
FIGURE 1. Histological characteristics evaluated in the study: A) koilocytotic-like change; B) necrosis; C) moderate to marked pleomorphism; D) individual keratinization, keratin pearls, and highly differentiated cells; E) invasive front in nests; F) diffuse invasive front. Hematoxylin and eosin staining.
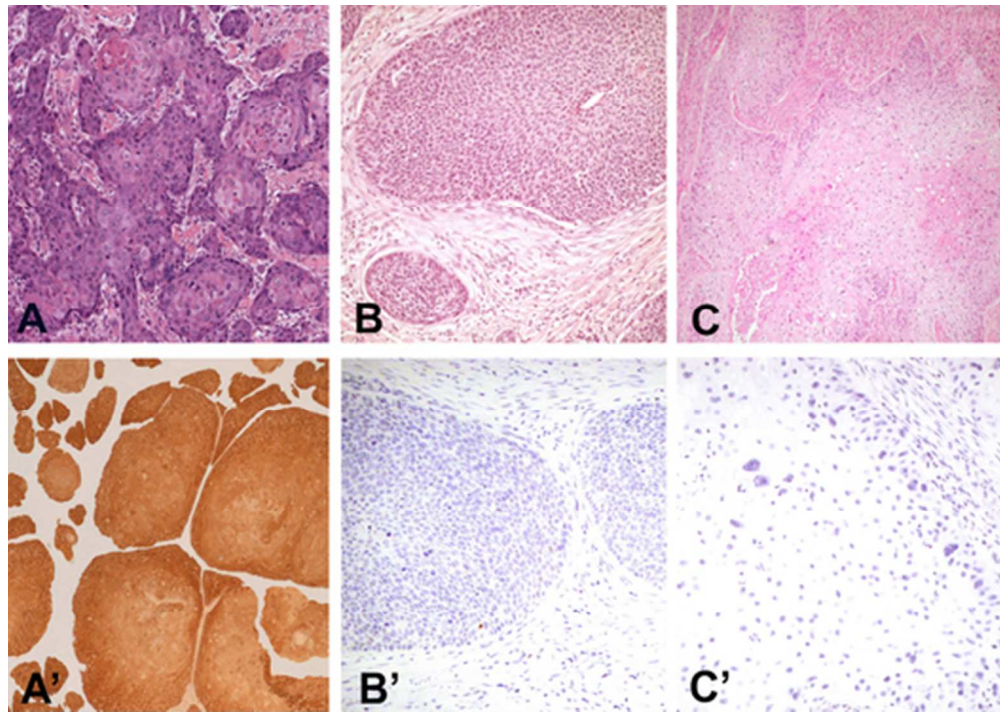
22x15mm (600 x 600 DPI)

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').

22x15mm (600 x 600 DPI)