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Contribution of Human papillomavirus in neuroendocrine tumors from a series of 10,575 invasive cervical cancer cases

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ABSTRACT

Aims: Neuroendocrine tumors (NET) of the cervix are rare tumors with a very aggressive course. The human papillomavirus (HPV) has been linked to its etiology. The objective of this study is to describe HPV prevalence and genotype distribution of NET.

Methods and Results: Forty-nine tumors with histological neuroendocrine features were identified among 10,575 invasive cervical cancer (ICC) cases from an international study. HPV DNA detection was done using SPF10/DEIA /LiPA₂₅ system. Immunohistochemical (IHC) staining for neuroendocrine markers (chromogranin A, synaptophysin, CD56) and for p16^{INK4a} as a surrogate for HPV transforming infection was performed. In 13 samples with negative IHC for all 3 neuroendocrine markers studied, it was possible to conduct electron microscopy (EM).

NET represented 0.5% of the total ICC series and HPV was detected in 42 out of 49 samples (85.7%, 95%CI:72.8%,94.1%). HPV16 was the predominant type (54.8%), followed by HPV18 (40.5%). p16^{INK4a} overexpression was observed in 38/44 cases (86.4%). Neuroendocrine IHC markers could be demonstrated in 24/37 (64.9%) cases. EM identified neuroendocrine granules in 8 samples with negative IHC markers.

Conclusions: Our data confirms the association of cervical NET with HPV and p16^{INK4a} overexpression. Specifically, HPV16 and 18 accounted together for over 95% of the HPV positive cases. Current HPV vaccines could largely prevent these aggressive tumors.

1. Introduction

Neuroendocrine tumors (NET) of the cervix are rare, accounting for 0.5–1% of all cervical carcinomas [1]. Since 1972, when the first study of carcinoid tumor of the cervix was published by Albores-Saavedra [2,3], some small series have been reported. Currently, the World Health Organization (WHO) includes 4 types of NET: carcinoid (C),

atypical carcinoid (AC), small cell carcinoma (SmCC) and large cell neuroendocrine carcinoma (LCC) [1]. However, terminology to categorize cervical NET has not always been clear [4] and diagnostic criteria are not very well defined, particularly large cell carcinomas [1] can be difficult to distinguish from poorly differentiated squamous carcinomas or adenocarcinomas. Neuroendocrine carcinoma of the cervix has a poor prognosis, with a high tendency to relapse and its

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precise diagnosis is of clinical relevance [5,6]. This is related to the fact that 90% of the cases present lymph-vascular invasion which is poor prognosis finding in most malignant tumors. Therefore, comparative studies of frequency, histological features, clinical behavior and the natural history of this group of tumors are limited.

The association between squamous cell carcinoma (SCC), adenocarcinoma (ADC) or adenosquamous carcinoma (ADSCC) of the cervix with human papillomavirus (HPV) has been well established and documented [7–9]. Overexpression of p16^{INK4a}, a cyclin dependent kinase inhibitor, is associated with high risk HPV due to E7 mediated inactivation of retinoblastoma tumor suppressor, and a negative feedback loop. p16^{INK4a} immunohistochemistry shows positive staining in nearly all squamous cell carcinomas (97%) and in about half of adenocarcinomas (48–50%) [10,11]. The expression of this protein, p16^{INK4a}, in NET of the uterine cervix is not well established.

According to published data, HPV detection in NET tumors ranged between 0 and 100%, with HPV18 and 16 being the most frequently detected genotypes [5,12–28]. The use of techniques with different sensitivity to detect HPV DNA, the inclusion criteria of the samples in each study and total cases included, and different methods of tissue conservation in the different participant centers may be the explanation for this wide range of results among series.

We present HPV DNA detection and genotype distribution, together with the histological and immunohistochemical features in 49 NET of the cervix.

2. Materials and Methods

2.1. Study design and materials

The Catalan Institute of Oncology (ICO) in Barcelona, Spain and the DDL Laboratories in Rijswijk, The Netherlands, have conducted an international study on HPV DNA detection in invasive cervical cancer (ICC) cases as previously described [8]. This study included 10,575 ICC cases from pathology archives from 38 countries of 5 continents diagnosed from 1940 to 2009. Participant centers provided consecutive cases diagnosed as ICC with basic information (country, histological diagnosis, age at diagnosis and year of diagnosis). After reviewing the histological diagnosis for these 10,575 cases by a team of 3 pathologists from ICO, 54 NET were identified. Five cases were excluded from the present study since the diagnosis was performed at the center of origin and not confirmed at ICO (unavailable Haematoxylin and Eosin-HE slide), leaving 49 NET cases confirmed by the ICO panel of pathologists using the criteria described by Albores-Saavedra et al. [3]. An algorithm of the study and procedures performed is shown in Fig. S1 of the supplementary material.

2.2. Tissue processing and histological diagnosis

Tissue processing and pathology diagnosis were done by the reference pathology laboratory at ICO. Paraffin blocks were processed under strict conditions to prevent contamination. First and last sections were used for histopathological evaluation on HE slides while intermediate sections were used for HPV DNA detection and genotyping. In addition, a blank paraffin section was cut between specimens to prevent any carry over contamination. Pathology evaluation was done from an HE slide of one tissue block and included: confirmation of ICC, histological classification, quantitation of tumor necrosis, amount of invasive tumor in the tissue section and presence of normal mucosa and/or preneoplastic lesion.

WHO classification of NET of the uterine cervix was used to subcategorize the cases as C-Carcinoid, AC-Atypical carcinoid, LCC-Large cell neuroendocrine carcinoma, SmCC-Small cell carcinoma, categories. SmCC and LCC carcinomas were distinguished from C and AC taking into account architectural and cytological features. Infiltrative margins, high mitotic rate (> 10/10 high power fields) and extensive geographic

areas of necrosis ruled out C and AC. Small cells with scant cytoplasm and the presence of compacting artifact were criteria to diagnose SmCC. LCC was diagnosed on the basis of chromatin features and larger cytoplasm compared to SmCC.

2.3. HPV testing

For each specimen, a paraffin tissue section was treated with 250 µl of freshly prepared Proteinase K solution to extract DNA. SPF-10 polymerase chain reaction (PCR) was performed using 10 µl of the DNA extract with a 1:10 dilution in a final reaction volume of 50 µl. The amplified PCR products were tested using a probe hybridization step with a cocktail of conservative probes, recognizing at least 54 mucosal HPV genotypes, in a microtiter plate format for the detection of HPV DNA through DNA Enzyme Immunoassay (DEIA). Optical densities (OD450) were read on a microtiter plate reader and categorized as HPV DNA negative, positive, or borderline. After PCR, 10 µl of the amplimers DEIA HPV DNA positive were used to perform the reverse hybridization line probe assay (LiPA₂₅) (version 1: produced at Laboratory Biomedical Products, Rijswijk, The Netherlands) [29]. LiPA₂₅ detects 25 high-risk (HR) and low risk (LR) HPV types (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74). The sequence variation within the SPF-10 primers allows the recognition of these different HPV genotypes, except for types 68 and 73, as their inter-primer regions are identical and cannot be distinguished by this test. Positive hybridization on the strips is visualized as a purple band by means of a precipitating color substrate on the probe site.

2.4. Immunohistochemistry

Immunohistochemical (IHC) staining was performed with the automated system Autostainer Link 48 (Dako Co, Carpinteria, CA) using primary antibodies to chromogranin (clone DAK-A3 DAKO), synaptophysin (clone 5438 DAKO) and CD56 (clone 123c DAKO), following the manufacturer's recommendations. Immunohistochemical neuroendocrine differentiation was considered when at least one of the three markers depicted cytoplasmic or membrane positive staining in tumor cells, even if it was focally.

As a surrogate of a transforming high risk HPV infection p16^{INK4a} was detected using the CINtec histology kit (clone E6H4, mtm Laboratories, Heidelberg, Germany), following the manufacturers protocol. A pattern of diffuse nuclear and cytoplasmic staining (> 50%) was considered a positive result.

In each run a negative and a positive control were included. These markers were performed when there was available material. Details on available material by marker are shown in Fig. S1 of the supplementary material.

2.5. Electron microscopy

Electron microscopy (EM) was performed in 16 cases, 13 cases with negative IHC for all the neuroendocrine markers performed, and 3 with IHC positive results used as controls. Appropriate areas were selected from original paraffin blocks and sampled with a tissue microarray needle (1 mm in diameter), deparaffined, post fixed in osmium tetroxide and embedded in epoxy resin. Sections were examined with a Phillips (FEI) CM 100 transmission electron microscope. The presence or absence of cell junctions and neuroendocrine granules in well preserved samples was reported.

2.6. Statistical analysis

Data analysis was performed with STATA version 10.0 (Stata Corporation, Computing Resource Center, College Station, Texas).

A descriptive analysis was performed by calculating proportions and 95% Confidence Interval-95%CI for qualitative variables and central

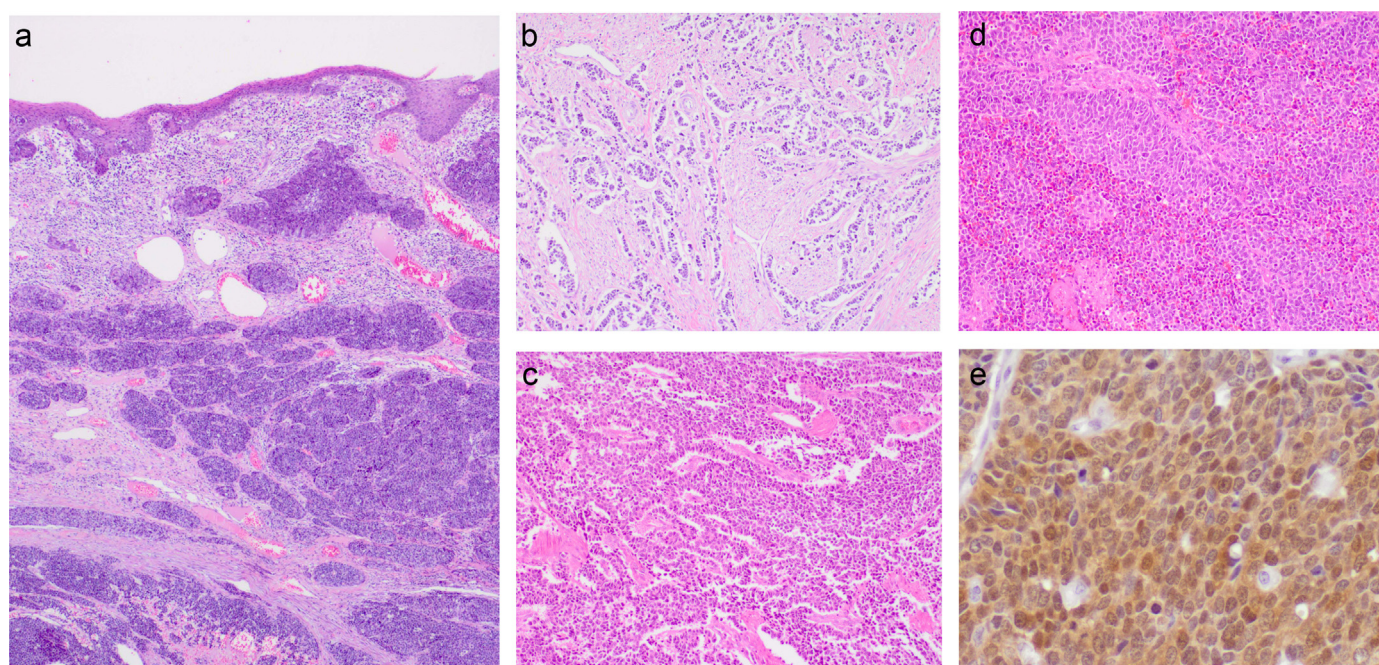


Fig. 1. Images of Hematoxylin & Eosin staining and p16^{INK4a} in neuroendocrine tumors. - (a) Small cell carcinoma. Low power of small cell carcinoma with a nesting pattern; (b) Small cell carcinoma; (c) Atypical carcinoid; (d) Large cell neuroendocrine carcinoma; (e) Neuroendocrine tumor with strong intracytoplasmic and intranuclear staining with p16^{INK4a}.

tendency and variability measures for quantitative variables. One-way ANOVA was used for comparison of age at diagnosis by histology, and Chi-Squared test for comparing HPV positivity by histological major groups. We assessed levels of similarity concordance between the HPV test and p16^{INK4a} by Kappa score. McNemar chi-squared test for matched pair data was used for assessing unequal distribution of discordant results. Significance for all analyses was set at the 2-sided 0.05 level.

HPV positivity refers to the percent positive cases among all HPV analyzed cases. HPV type-specific relative contribution refers to the percent positive for a given type, related to all HPV positive samples. Multiple-infected cases were counted as follows: 1) As one case (combination of types) in Table 2; 2) Adding multiple infections to single types under a proportional weighting attribution in Fig. 2 [8], and 3) Counting infections not cases, this is each HPV type as one infection either in single and multiple infections in Table 3.

2.7. Ethical issues

All protocols were approved by local and ICO ethics committees and

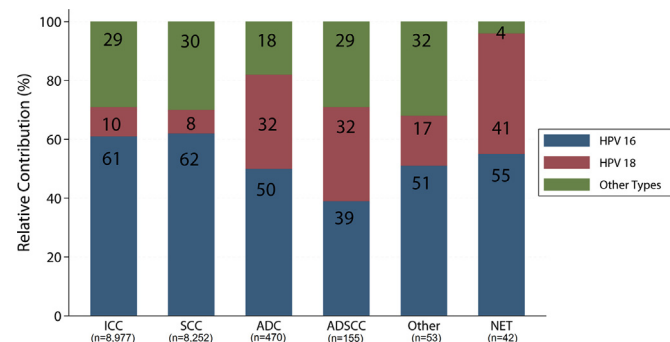


Fig. 2. HPV type distribution among HPV positive cases by histological classification. “ICC”-Invasive Cervical Cancer; “SCC”-Squamous Cell Carcinoma; “ADC”-Adenocarcinoma; “ADSCC”-Adenosquamous Cell Carcinoma; “OTHER”-Mostly undifferentiated cases; “NET”-Neuroendocrine Tumors. (*) Other category, does not contain NET cases.

the study progress was overseen by an international steering committee.

3. Results

3.1. Cases

Among 10,575 cervical carcinomas, 49 NETs were identified from histology, representing 0.5% of the total series. Table 1 describes the distribution of the cases by region and time of diagnosis. We comparatively observed no significant differences either within or between NET and various other tumors types, which were compared, considering different regions and times of diagnosis. The mean age at diagnosis of NET was 51.8 years (Standard Deviation: 15.0 years).

In accordance with the WHO classification of NET cases were subcategorized as C (N=1), AC (N=4), LCC (N=11) and SmCC (N=33) (Fig. S1 of supplementary material). In 7 cases there was a second component identified: 5 carcinomas contained areas of ADC, one showed SCC, and another undifferentiated carcinoma.

3.2. HPV Analysis

HPV DNA was detected in 85.7% (95%CI: 72.8%, 94.1%) of NET (42/49), in concordance with results of the whole ICC series (84.2%, 95%CI: 84.2%, 85.6%; p-value > 0.05) [8]. 97.6% of the HPV positive cases were infected only with one HPV type (single infection) and one SmCC case was infected by 2 HPV types (HPV18&HPV52) (Table 2). Fifty five percent of NET cases were HPV16 positive; 41% were positive for HPV18 and 4% were positive for other types (Fig. 2). The genotype distribution by histology showed a statistically significant higher proportion of HPV18 in NET cases compared to SCC and other histologies.

3.3. Immunohistochemistry for NET differentiation

All three immunohistochemical markers (chromogranin, CD56 and synaptophysin) could be performed in 37 out of 49 cases (Fig. 3 and Table 2) and 64.8% were positive for at least one marker. No tissue

Table 1

Number of invasive cervical cancer cases included for HPV DNA detection in the entire series [8] by region and time at diagnosis, stratified by histological categories.

	ICC cases HPV analyzed N	SCC N (%)	ADC N (%)	ADSCC N (%)	OTHER N (%)	NET ^(*) N (%)
Region						
Europe	2,364	2,093 (88.5)	209 (8.8)	43 (1.8)	12 (0.5)	7 (0.3)
North America	176	160 (90.9)	13 (7.4)	0 (0.0)	2 (1.1)	1 (0.6)
Latin America	4,171	3,764 (90.2)	261 (6.3)	80 (1.9)	45 (1.1)	21 (0.5)
Africa	691	609 (88.1)	58 (8.4)	17 (2.5)	4 (0.6)	3 (0.4)
Asia	2,994	2,722 (90.9)	197 (6.6)	39 (1.3)	19 (0.6)	17 (0.6)
Oceania	179	138 (77.1)	22 (12.3)	12 (6.7)	2 (0.8)	0 (0.0)
Time at diagnosis						
1940–1949	114	99 (86.8)	11 (9.6)	2 (1.8)	1 (0.9)	1 (0.9)
1950–1959	533	483 (90.6)	36 (6.8)	7 (1.3)	5 (0.9)	2 (0.4)
1960–1969	979	909 (92.8)	50 (5.1)	14 (1.4)	5 (0.5)	1 (0.1)
1970–1979	1,322	1,171 (88.6)	113 (8.5)	23 (1.7)	6 (0.5)	9 (0.7)
1980–1989	1,499	1,370 (91.4)	83 (5.5)	32 (2.1)	7 (0.5)	7 (0.5)
1990–1999	2,748	2,451 (89.2)	213 (7.8)	49 (1.8)	21 (0.8)	9 (0.3)
2000–2009	3,380	3,003 (88.8)	254 (7.5)	64 (1.9)	39 (1.2)	20 (0.6)
TOTAL	10,575	9,486 (89.7)	760 (7.2)	191 (1.8)	84 (0.8)	49 (0.5)

“ICC”-Invasive Cervical Cancer; “SCC”-Squamous Cell Carcinoma; “ADC”-Adenocarcinoma; “ADSCC”-Adenosquamous Cell Carcinoma; “OTHER”-Mostly undifferentiated cases without NET cases; “NET”-Neuroendocrine Tumors; %: Row percentages.

(*) Five cases were excluded since histological diagnosis was performed in the centre of origin and slides were not retrieved to the reference lab for confirmation. The total number of confirmed histological NET diagnosis were 49 out of 54 initially NET cases.

from the carcinoid NET was available on which to perform IHC NET markers. Three AC displayed positive staining for all 3 markers, LCC showed at least one positive marker in 7 cases (70.0%) and 3 cases were completely negative. Among SmCC, 10/24 (41.7%) were positive for at least one IHC NET marker. When analyzed individually each marker, CD56 was the most frequently positive immunohistochemical marker 61.5% (24/39) followed by chromogranin 38.5% (15/39) and synaptophysin was detected in 25.6% (10/39) of the cases.

3.4. Electron microscopy (EM)

Three out of 13 cases showed poor preservation and were considered inadequate for EM interpretation. Primitive cell junctions were found in 6 cases and 5 showed desmosomes. Neurosecretory-type granules, with diameters ranging from 60 to 339 nm, were seen in 8

cases (Fig. 3). Overall, EM confirmed neuroendocrine differentiation in 8 (2 LCC and 6 SmCC) out of 10 cases that were IHC negative for all available markers (Table 2). All positive controls showed neurosecretory granules.

3.5. p16^{INK4a} immunohistochemistry

p16^{INK4a} staining could be performed in 44 cases and 86.4% (95%CI: 72.6%,94.8%) showed over-expression (Table 2). All C, AC and LCC were p16^{INK4a} positive (Fig. 1), while 22 of 28 (78.5%) SmCC showed p16^{INK4a} positive staining. All p16^{INK4a} negative cases showed poor tissue preservation. Overall, concordant results of p16^{INK4a} and HPV detection was observed in 88.6% of the cases (Kappa Index: 0.549, p-value < 0.001, McNemmar test p-value > 0.05).

Table 2Immunohistochemistry, electron microscopy results, p16^{INK4a} overexpression and HPV DNA detection by neuroendocrine subtype histological classification.

	NET cases	Neuroendocrine markers							HPV DNA detection and related markers						
		NA	Chrom	Synap	CD56	IHC neg*	EM NE + **		p16 ^{INK4a}	HPV positivity	HPV16 Single	HPV18 Single	HPV35 Single	HPV58 Single	Multiple infections
NET Classification	N	(%)^a	N	n/N	n/N	n/N	n/N	n/N	n/N (%)^b	n/N (%)^c	N (%)^d	N (%)^d	N (%)^d	N (%)^d	(N; %)^d
Carcinoid	1	(2.0)	1	-	-	-	-	-	1/1 (100.0)	1/1 (100.0)	1 (100.0)	-	-	-	-
Atypical carcinoid	4	(8.2)	1	3/3	3/3	3/3	0/3	-	4/4 (100.0)	2/4 (50.0)	-	2 (100.0)	-	-	-
Large cell carcinoma	11	(22.4)	0	4/11	8/11	6/10	3/10	2/2	11/11 (100.0)	10/11 (90.9)	7 (70.0)	2 (20.0)	-	1 (10.0)	-
Small cell carcinoma	33	(67.3)	7	8/25	7/25	15/26	10/24	6/8	22/28 (78.6)	29/33 (87.9)	15 (51.7)	12 (41.4)	1 (3.4)	-	HPV18&52 (1; 3.4)
TOTAL	49 (100.0)		9	15/39	10/39	24/39	13/37	8/10	38/44 (86.4)	42/49 (85.7)	23 (54.8)	16 (38.1)	1 (2.4)	1 (2.4)	HPV18&52 (1; 2.4)

"NET"-Neuroendocrine Tumors; "HPV"-Human Papillomavirus;"DNA"-Deoxyribonucleic Acid;"NA"-Not assessable/available for immunohistochemistry - IHC; "Chrom"-Chromogranin; "Synap"-Synaptophysin;

* Immunohistochemistry markers negative (All 3 immunohistochemical markers performed and with a negative result);

** "EM NE+": Neuroendocrine differentiation on electron microscopy study (Only performed in cases with all available immunohistochemistry (IHC) markers negative);

a) N = number of cases, % = percentage over total neuroendocrine tumors (NET); b) n = number of cases with a positive result for the specific immunohistochemistry (IHC) marker, N = number of cases analyzed for the specific IHC marker, %: n/N; c) n = number of cases HPV positive, N = number of cases HPV analyzed, %: n/N; d) HPV types %: number of cases positive for a specific HPV type among the HPV positive cases.

NOTE: p16^{INK4a} was not performed in all cases, due to lack of tissue.

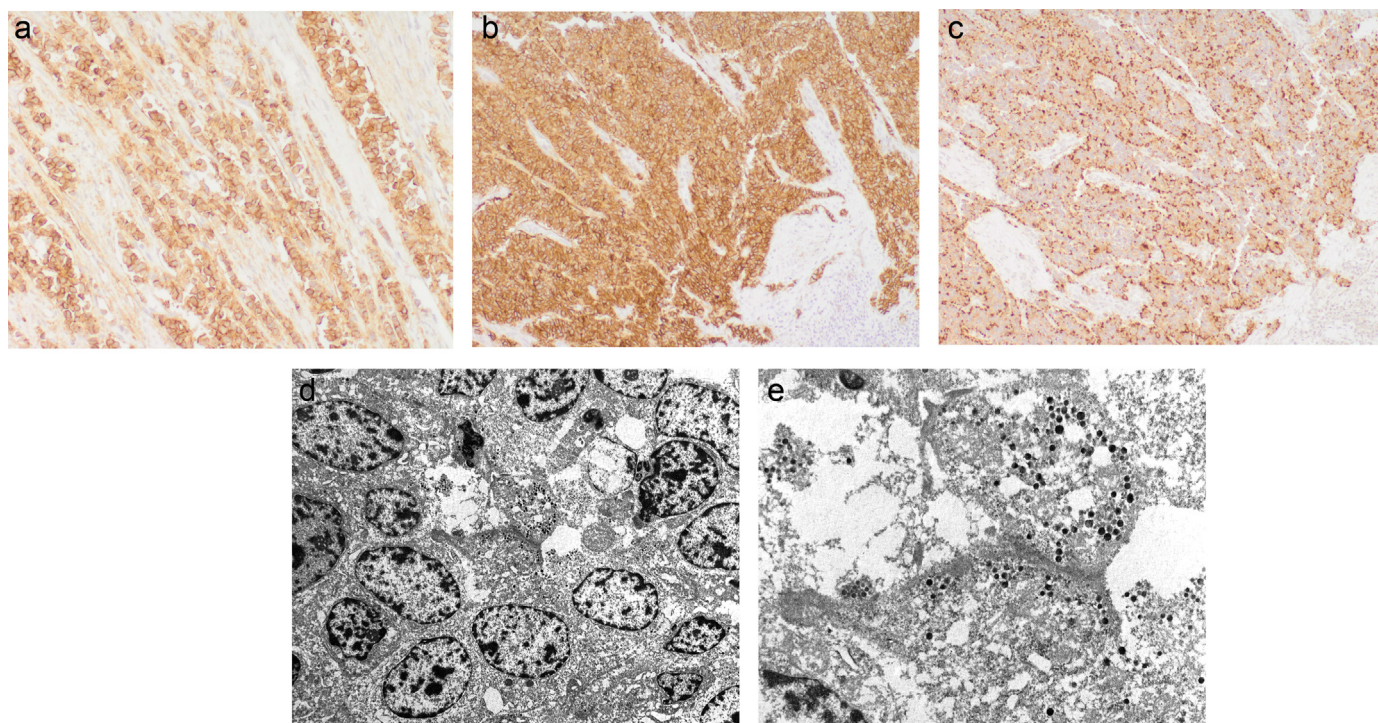


Fig. 3. Neuroendocrine differentiation evaluated by immunohistochemistry and electron microscopy. (a) Diffuse immunostaining for CD56 and (b) staining for synaptophysin confirming the presence of neuroendocrine differentiation; (c) Diffuse staining for chromogranin; (d) Electron microscopic picture showing tumor cells arranged concentrically in a rosette-like configuration, with a moderate amount of cytoplasm and round to ovoid nuclei, with a characteristic pattern of combined euchromatin and heterochromatin, occasional small nucleoli and slight irregularities in the nuclear membrane outlines (Original magnification: 2600 $\times$); (e) Inset: Detail of two cell processes containing numerous electron dense neuroendocrine granules, with diameters ranging from 71 nm to 181 nm. Primitive cell junctions can be seen between them (Original magnification: 7900 $\times$).

4. Discussion

From a large international study of invasive cervical carcinoma, 49 NET were identified, which represents 0.5% of the total. This figure is consistent with the low frequency (0.5–1%) previously reported in the literature [1]. HPV DNA was detected in a vast majority of NET cases (85.7%) confirming its role as in other cervical carcinoma histologies, and reveals an almost equal contribution of HPV16 and HPV18 in its etiology (HPV16 in 54.8% and HPV18 in 40.5%), while other HPV types were rarely detected. In this series, HPV18 was four times (40.5%) more frequent in NET than in the whole series of ICC (10.1%) (p-value < 0.001). HPV type distribution in NET parallels what is found in ADC and ADSCC. Previous data showed that 81.8% of the NET was positive for HPV, with HPV18 detected in 73.1% and HPV16 in 30.8% (Table 3) [5,12–28]. Percentage of HPV18 detection ranged from between 22% to 100%. This wide range can be due mainly to two causes. The variability of the sample size on previous published data: more than half of the papers were based on less than 20 cases. On the other hand, LCC subtype can be easily confused with a poorly differentiated squamous cell carcinoma based on the identification of focal areas of squamous differentiation when no immunohistochemical markers are used. This hypothesis is supported by the fact that most series include mainly small cell neuroendocrine carcinomas and only few cases of LCC. Grayson et al. [20] who also included LCC, detected 77.8% HPV16 and 22.2% HPV18. Therefore, probably in SmCC the contribution of HPV18 is greater than in LCC.

The overall distribution of HPV types in cervical cancer has not changed significantly over time. No time trend for SCC has been observed; meanwhile in ADC has observed an increasing trend for HPV16 and a decreasing trend for HPV18 [30,31]. This may be due to a wider coverage of cytology based screening programs allowing for detection of concurrent lesions associated (i.e., joint detection of ADC and

squamous intraepithelial lesions, in large part HPV16 positive). In our study, the number of NET detected cases is small and we cannot assess any time trend. Broad series that include all subtypes of NETs are needed to see if there has been any change over time.

A common occurrence of NET with premalignant or invasive glandular cervical lesions has been reported [26,32,33] and neuroendocrine cells have been detected in normal endocervix [34] suggesting that NET and ADC could have a similar origin or carcinogenic pathway. The greater frequency of HPV18 in both NET and ADC observed in our series and in other histologies (Table 3) is suggestive of greater affinity of HPV18 for glandular and neuroendocrine cells as compared to other HPV types. Indeed, in our study NET was more frequently associated with concomitant glandular than with squamous lesions.

Within NET, SmCC was the most frequent subtype identified (67.3%), while 22.4% of the cases were LCC. The lower frequency of LCC could be related to a more difficult differential diagnosis between LCC and squamous cell carcinomas, poorly differentiated ADC or undifferentiated carcinomas, taking into account that immunohistochemical neuroendocrine markers are not performed as a routine staining [20,32–35]. The issue becomes relevant because under the same clinical stage, prognosis is worse for NET than for SCC or ADC of the cervix [5,6]. High clinical aggressiveness, frequent local recurrences and distant metastases observed in NET [36,37] has been demonstrated by a marked lymphatic permeation that occurs in these tumors; a feature which is particularly characteristic of HPV18 related tumors [27,37]. Unfortunately we could not evaluate the evolution of our cases due to study design limitations, as no follow up data was collected.

The diagnosis of NET is made primarily by the examination of conventional HE sections. Immunostaining with chromogranin A, synaptophysin and CD56 can help to establish the correct diagnosis

Table 3
Literature review; published papers regarding HPV detection in neuroendocrine tumors (n > 1 case).

Author (year) [Ref]	Cases included in the study (N)	Histological diagnosis (N)	HPV detection technique (cases HPV analyzed)	HPV types analyzed	HPV + n (%) ^a	HPV16 n (%) ^b	HPV18 n (%) ^b	Other HPV types (n; %) ^b	Multiple HPV types (n; %) ^b	p16 (+) (%)	Neuroendocrine differentiation IHC markers (% positivity at least 1 marker)
Pao C.C. et al. (1991) [12] [*]	12	SmCC (12)	PCR (DNA) (in all cases)	16,18,31,33	0 (0.0)	-	-	-	-	-	-
Stoler M.H. et al. (1991) [13]	20	SmCC (20)	ISH (mRNA) (in all cases)	6,11,16,18,31	17 (85.0)	3 (17.6)	14 (82.4)	-	-	-	NSE; Chrom; Synap (18/20 = 90%)
Ambros R.A. et al. (1991) [14]	7	SmCC (7)	PCR (DNA) (in all cases)	16,18	4 (57.1)	1 (25.0)	3 (75.0)	-	-	-	NSE; Chrom; Synap; Ser (7/7 = 100.0%)
Abeler V.M. et al. (1994) [15]	26	SmCC(12) LCC (14)	ISH (DNA) (in 25 cases)	6,11,16,18	17 (68.0)	7 (41.2)	10 (58.8)	-	-	-	NSE; Chrom; Synap; Leu7 (19/24 = 79.2%)
Mannion C. et al. (1998) [16]	38	SmCC (25) LCC (5) C (5)	ISH (DNA) (in 10 cases)	6/11,16/18, 31/33	8 (80.0)	-	-	-	HPV16/18 (5; 62.5) HPV31/33/ 16/18 (3; 37.5)	-	-
Wisnuba I.I. et al. (1999) [17]	15	Mix SmCC/LCC (3) SmCC(8) LCC (2) C (1) AC (4)	PCR (DNA) (in all cases)	16,18,31,33	8 (53.3)	2 (25.0)	6 (75.0)	-	-	-	-
Herrington C.S. et al. (1999) [18]	25	SmCC (25)	PCR (DNA) (in all cases)	6,11,16,18,31,33, 35,42,43,44,45, 51,52,56,58,66	25 (100.0)	13 (52.0)	11 (44.0)	HPV45 (1; 4.0)	-	-	Chrom; Synap (100.0%)
Shiyr J.S. et al. (2001) [19]	3	SmCC (3)	ISPCR (DNA) (in all cases)	16,18	3 (100.0)	1 (33.3)	1 (33.3)	-	HPV16&18 (1; 33.3)	-	-
Grayson W. et al. (2002) [20]	12	LCC (12)	PCR (DNA) (in all cases)	16,18,31,33,35, 52,58	9 (75.0)	7 (77.8)	2 (22.2)	-	-	-	Chrom; Synap (11/12 = 91.7%)
Masumoto N. et al. (2003) [21]	10	SmCC (10)	PCR (DNA) (in all cases)	16,18,31,33	10 (100.0)	1 (10.0)	9 (90.0)	-	-	100.0%	NSE; Chrom; Synap; So; Ser (9/10 = 90.0%)
Ishida G.M. et al. (2004) [22]	10	SmCC (5) Mix SmCC/LCC (5)	PCR (DNA) (in all cases)	6,11,16,18,31,33,42,52,58	8 (80.0)	-	8 (100.0)	-	-	-	Chrom; Synap; So; Ser (100.0%)
Wang H.L. et al. (2004) [23]	22	SmCC (22)	PCR (DNA) (in all cases)	25 HPV types: INNO-LiPA	22 (100.0)	4 (18.2)	17 (77.3)	-	HPV16&18 (1; 4.5)	91.0%	NSE; Chrom; Synap (100.0%)
Horn L.C. et al. (2006) [24]	9	SmCC (9)	PCR (DNA) (in all cases)	20 HPV types: GP5+ /GP6(+)	8 (88.9)	4 (50.0)	1 (12.5)	HPV3 (1; 12.5) HPV68 (1; 12.5) HPV90 (1; 12.5)	-	100.0%	NSE; Chrom; Synap; CD56 (100.0%)
Wang K.L. et al. (2006) [5] [*]	31	SmCC (25) LCC (4) AC (2) LCC (7)	Not available (in 26 cases) PCR (DNA) (in all cases)	Not available 16,18	18 (69.2) 6 (85.7)	1 (5.6) -	17 (94.4) 6 (100.0)	-	-	-	NSE; Chrom; Synap (100.0%)
Wang K.L. et al. (2009) [25]	7	SmCC (13) LCC (8)	PCR (DNA) (in 2 cases)	37 HPV types: Linear Array	2 (100.0)	1 (50.0)	1 (50.0)	-	-	95.2%	Chrom; Synap; CD56; PGP9.5 (100.0%)

(continued on next page)

Table 3 (continued)

Author (Year) [Ref]	Cases included in the study (N)	Histological diagnosis (N)	HPV detection technique (cases HPV analyzed)	HPV types analyzed	HPV + n (%) ^a	HPV16 n (%) ^b	HPV18 n (%) ^b	Other HPV types (n; %) ^b	Multiple HPV types (n; %) ^b	p16 (+) (%)	Neuroendocrine differentiation IHC markers (% positivity at least 1 marker)
Siriakungul S. et al. (2011) [27]	111	SmCC (89)	PCR (DNA)	16,18,31,33, 35,39, 45,51,52,56,58,59,66,68	93	15	57	HPV33 (1; 1.1)	HPV16&18 (11; 11.8) HPV16&35 (1; 1.1) HPV18&52 (1; 1.1)	-	Selection criteria for non-SmCC: at least 1 positive of Chrom; Synap; CD56
Shiho Kuji et al. (2016) [28]	37	LCC (5) AC (3)	(in 97 cases)		(95.9)	(16.1)	(61.3)	HPV58 (3; 3.2) HPV16 (3; 3.2)	HPV18&52 (1; 1.1) HPV16&18& 52 (1; 1.1)	-	
TOTAL	-	-		6,11,16,18,31,33, 35,39, 45,51,52,56,58,59,66 HPV positivity: 279/341 (81.8%) ^a	21 (72.4)	3 (14.3)	18 (85.7)	-	-	-	INSM1 (95%); Chrom (86%); Synap (86%); CD56: 68%

“AC”- Atypical Carcinoid; “C”- Carcinoid; “Chrom”- Chromogranin; “IHC”- Immunohistochemistry; “ISH”- In Situ Hybridization; “ISPCR”-In Situ Polymerase Chain Reaction; “LCC”- Large Cell Carcinoma; “NSE”- Neuron Specific Enolase; “PCR”- Polymerase Chain Reaction; “SmCC”- Small Cell Carcinoma; “Ser”- Serotonin; “So”- Somatostatin; “Synap”- Synaptophysin; “INSM1”- Insulinoma-associated protein 1.
a) HPV positivity %: number of cases HPV positive among HPV analyzed cases; b) HPV types %: number of cases positive for a specific HPV type among the HPV positive cases; c) Global HPV type-specific distribution: number of cases positive for a specific type (multiple infections count once) among HPV positive cases; *Only abstract available.

[26,37]. In our series, IHC markers of neuroendocrine differentiation were detected in 65.8% of the tumors in which the three markers could be performed. Albores Saavedra [38] and McCluggage [26] found CD56 to be the most sensitive marker of neuroendocrine differentiation (61.5%) followed by chromogranin A (38.5%) and synaptophysin (25.6%). In published series, immunoreactivity for chromogranin and synaptophysin ranges from 50% to 100% [16,17]. The negativity of these markers observed in our study could be attributed to differences in the processing of tissue, some of them being very old, as 13 cases were diagnosed before 1980 when buffered formalin was introduced in pathology laboratories to optimize immunohistochemical staining. The cases diagnosed before 1980 were more frequently negative (91.7%) in Chromogranin marker than the cases diagnosed after 1980 (48.1%) (Fisher's exacts p-value test < 0.02). However, most tumors that did not show NET markers by IHC were SmCC, which harbor the most typical histological features. In 6 SmCC with negative IHC markers, EM showed neurosecretory granules. Therefore, by adding EM as a diagnostic tool we reached a confirmation of neuroendocrine differentiation in 32 out of 49 cases (65.3%). Immunohistochemistry can be very helpful in confirming a diagnosis of NET but should not be used to disregard a morphological diagnosis.

Diffuse and continuous, cytoplasmatic and nuclear, expression of p16^{INK4a} is an excellent immunochemical surrogate for SCC harboring transforming high risk HPV due to HPV E7 mediated inactivation of retinoblastoma tumor suppressor, and a negative feedback loop. p16^{INK4a} was found over-expressed in 86.4% of NET and showed good concordance with the HPV result. In previous reports, 91–100% [21,23,24] SmCC showed p16^{INK4a} positivity while in this series it was present in 78.6% of the cases. All of cases of atypical carcinoid and large cell neuroendocrine carcinoma were positive for p16^{INK4a} with a similar proportion to that which is observed among SCC [10,11].

Another limitation of our study is the proportion of samples in which HPV DNA was not detected, as this was observed in the overall study [8]. The different procedures in transportation and fixation of the samples among different laboratories may affect the rate of positive HPV DNA detected; however since many centers from many different countries were involved the analysis is not informative. No time trend was detected which could explain the differences. If one accepts this argument, all the cervical NET would be most likely HPV related tumors.

This is a large series analyzed by PCR including all histological neuroendocrine subtypes and the results confirm the role of HPV, in particular HPV16 and 18. This fact is remarkable in the field of prevention, since HPV vaccines currently available could prevent more than 95% of these tumors.

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Disclosure/Conflict of Interest

Suzanne M. Garland: has received board membership from GlaxoSmithKline, Commonwealth Serum Laboratories advisory board for cervical cancer vaccine; payment for development of education presentations from GlaxoSmithKline, EXCEL program; grants have been paid to SMG's institute by the Royal Women's Hospital Melbourne.

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Silvia de Sanjosé: has received occasional travel fund to attend scientific meetings from Merck, Sanofi Pasteur MSD and Qiagen; and unrestricted grants through the Institution to conduct epidemiological studies from Qiagen, GlaxoSmithKline and Merck.

Belen Lloveras: has received payment for lectures including service on speakers bureaus (personal) and financial support for attending scientific meetings from Roche and Qiagen.

All other authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.pvr.2018.03.005>.

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