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Diagnostic algorithmic proposal based on comprehensive immunohistochemical evaluation of 297 invasive endocervical adenocarcinomas

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Abstract

The International Endocervical Adenocarcinoma Criteria and Classification (IECC) was developed to separate endocervical adenocarcinomas (ECAs) into two main categories based on morphology as HPV-associated (HPVA) and non-HPV-associated (NHPVA) adenocarcinomas. We aimed to improve the diagnostic accuracy of IECC by performing a comprehensive immunohistochemical (IHC) evaluation and constructing objective IHC-based algorithms for the classification of these tumors.

Tissue microarrays (TMA) were constructed from 297 of 409 cases used to develop the original classification. Immunostains included: p16, p53, estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), Vimentin, CK7, CK 20, HER2, HIK1083, MUC6, CAIX, SATB2, HNF1beta, napsin A, PAX8, CDX2, GATA3, p63, p40 and TTF-1. High-risk HPV (HR-HPV) was detected by in situ hybridization (ISH) using probes against E6 and E7 mRNA expressed in 18 different virus types.

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Vimentin, ER and PR were expressed in a significant minority of ECAs, mostly HPVAs, limiting their use in differential diagnosis with endometrioid carcinoma when unaccompanied by HPV ISH or p16. HR-HPV ISH had superior sensitivity, specificity, negative and positive predictive values compared to p16, as published previously. HNF-1beta did not have the anticipated discriminatory power for clear cell carcinoma, nor did MUC6 or CA-IX for gastric-type carcinoma. HNF-1beta and napsin A were variably expressed in clear cell carcinoma, with HNF-1beta demonstrating less specificity as it was ubiquitously expressed in gastric-type carcinoma and in the majority of HPV-associated mucinous (predominantly intestinal-type and invasive ECA resembling stratified mucin-producing intraepithelial lesion [iSMILE]) and usual-type carcinomas. HIK1083 was expressed in nearly half of gastric-type carcinomas, but not in the vast majority of other subtypes. GATA3 was positive in 10% of usual-type adenocarcinomas and in single examples of other subtypes. Rare gastric-type and HPVA mucinous carcinomas displayed HER2 overexpression. AR was positive in 6% of usual-type adenocarcinomas. Aberrant p53 expression was found in only 3.6% of usual-type HPVA carcinomas, but it was more prevalent in mucinous (intestinal type and iSMILE) HPVAs and NHPVAs (particularly in gastric-type carcinoma, >50% of cases). The following diagnostic classification algorithms were developed with the above data. Carcinomas without overt cytoplasmic mucin (endometrioid, usual-type endocervical, clear cell and mesonephric carcinomas) can be subclassified using HR-HPV ISH, ER and GATA3, while carcinomas with easily appreciated cytoplasmic mucin (endometrioid carcinoma with mucinous features, HPVA-mucinous and gastric-type carcinomas) can be subclassified with HR-HPV ISH and ER.

Keywords

Endocervical adenocarcinoma; Cervical carcinoma: Immunohistochemistry; HPV; Human papilloma virus

Introduction

Invasive ECAs are currently classified based on subjective descriptive morphological characteristics, particularly cytoplasmic features,[1] assessed on hematoxylin-eosin (H&E) stained slides. This has led to heterogeneous categories of ECAs that, with only occasional exceptions, are not useful in clinical management.[2]

A panel of pathologists from 7 different international institutions proposed a new classification scheme that separates ECAs into two main categories, using H&E slides: HPV-associated adenocarcinoma (HPVA), and non-HPV-associated adenocarcinoma (NHPVA).[3] Data from studies of vulvar squamous carcinoma and carcinoma in non-gynecological sites, such as head and neck, suggest that a classification based on pathogenesis is clinically informative and reproducible.[4–7] The validity of this new classification (International Endocervical Adenocarcinoma Criteria and Classification [IECC]) is supported by clinical data and HPV status.[3] Compared to HPVAs, NHPVAs present at a more advanced stage and are associated with more aggressive clinical behavior, with higher recurrence rates and worse overall survival.[8]

According to the IECC, the most frequent subtype in the HPVA category is usual-type ECA, 95% being HPV-positive, ascertained using a RNA-based in-situ hybridization assay that recognizes 18 different types of HR-HPV. In a recently published report, gastric-type, clear cell, endometrioid, serous and mesonephric carcinomas were HPV-negative.[3] Similar results have been reported by other investigators.[1,9–12] HPVA carcinomas include those lacking obvious intracytoplasmic mucin (i.e. usual-type) and those containing intracytoplasmic mucin, such as mucinous NOS, intestinal mucinous, signet ring cell and invasive ECA resembling stratified mucin-producing intraepithelial lesion (iSMILE). As both gastric-type carcinomas (NHPVAs) and HPVA-associated mucinous carcinomas contain intracytoplasmic mucin, recognition of the morphologic features associated with HPV infection (such as conspicuous floating mitoses and apoptosis), HPV in-situ hybridization and IHC can be used to refine the diagnosis.

Commonly encountered problems in differential diagnoses include distinguishing between the following: endometrioid and usual-type endocervical carcinoma; mucinous carcinomas of HPVA type and gastric-type carcinoma; clear cell and mesonephric carcinoma; clear cell and gastric-type carcinoma; primary endocervical and metastatic mucinous carcinoma; and endocervical and endometrial primary carcinoma. A recent abstract examining interobserver diagnostic concordance using the IECC system reported only fair interobserver agreement (K=0.33) among 7 experienced gynecologic pathologists (although majority agreement was achieved in 74% of 87 cases, with the highest levels of agreement reported for gastric-type and clear cell carcinomas).[13] Cases with majority agreement had excellent correlations with predicted HPV status, but the lowest levels of agreement were found with subtyping variants of HPVA tumors. These figures invoke the need for more precise biomarkers or combinations thereof for a meaningful and reproducible classification of invasive endocervical adenocarcinomas.

In an attempt to improve objective diagnostic accuracy, we performed a comprehensive IHC evaluation of a wide range of ECA types, as classified by the IECC.

Materials and Methods

Institutional approval for this study was obtained from each of the participating centers.

Patient selection

Slides from 409 invasive ECAs with at least 5-year follow-up were collected. In-situ carcinomas, squamous carcinomas, adenosquamous carcinomas, tumors with a neuroendocrine component, carcinosarcomas, and any tumor demonstrating clinical, macroscopic or microscopic features suggesting a lower uterine segment, uterine corpus, or adnexal primary were excluded. Tumors treated with neoadjuvant chemotherapy and/or radiotherapy were also excluded. Types of specimens included were: conizations/trachelectomies/hysterectomies and exenterations with lymph node dissection; however, biopsy and LEEP specimens were excluded.

Morphological assessment

All subtypes of ECA were included in this study. Assessment of morphology required examination of all H&E slides with tumor present (an average of 12 slides per case). A consensus diagnosis was reached in every case, with at least 2 and as many as 4 study pathologists reviewing slides at a multi-head microscope. Tumors were classified according to the new classification proposal (IECC)[8] (Figure 1; IECC criteria in Table 1).

Tissue microarray construction and Immunohistochemical study

Tissue microarrays (TMA) were constructed using previously described methods.[14,15] These included 297 cases from New York, Boston, Mexico, Japan and Romania to perform p16, p53, Progesterone receptor (PR), Androgen receptor (AR), Vimentin, HER2, HIK1083, MUC6, CAIX, SATB2, HNF1beta, PAX8, CK7, CK20, CDX2, GATA3, p63, p40 (Table 2). Each of the tumors from the New York, Mexico, and Romania centers was represented by three 0.6 mm cores, while cases from Japan were represented by single 3 mm cores. Except for ER, CK20, napsin A and GATA-3, which were scored by 1 pathologist (RAS or TK), stains were scored by 2 study pathologists (RAS and SS) reaching a consensus. Disagreements were extremely rare (approximately 2-3%) and were adjudicated by re-reviewing stated criteria for positivity, as described below. In some cases, only 1 or 2 cores remained on the stained slide; and were still considered eligible for scoring. p16 was interpreted as positive if diffuse, if block-like staining was found in all cores; no staining, or patchy staining, was interpreted as negative. p53 was scored as positive if ≥75% of tumor cell nuclei were strongly positive or if no staining was present in the background of an intact internal control. ER, PR, AR, PAX8, CK7, CK20, HNF-1beta and napsin A were interpreted as positive if >25% (score 3 or 4) of tumor cell nuclei or cytoplasm (CK7, CK20 and napsin A) were stained as follows: Score 0: <5%; score 1+: 5–10%; score 2+: 11–25%; score 3+: 26–75%; score 4+: more than 75%. Vimentin was scored as positive if ≥50% of tumor cells showed membranous/cytoplasmic staining. HER2 was scored using the CAP guidelines for gastric carcinoma: 3+ membranous positive.[16] HIK 1083, MUC 6 and CAIX, SATB2, GATA3, p63, p40 and CDX2 were considered positive if any nuclear staining was noted in >5% of tumor cells. HIK 1083 is currently not available in the US.

HPV detection

HPV detection for HR-HPV subtypes was performed on all ECAs in the TMA that had sufficient tissue to score, and had not been improperly fixed or stored (n=168). HPV in-situ hybridization with a chromogen was performed using the Advanced Cell Diagnostics (ACD) (Hayward, CA) RNAscope® system (catalogue no.312598). The RNAscope® Probe “HPV HR18” contains probes targeting E6 and E7 mRNA for the following high-risk subtypes: HPV16,18,26,31,33,35,39,45,51,52,53,56,58,59,66,68,73 and 82. The methodology and interpretation were discussed in detail in a previous paper.[17]

Statistical analysis

Standard statistical methods were used, including analysis of variance, utilizing the statistical package programs STATA 13 (StataCorp).

Results

P16 and HPV-ISH

These data were recently reported.[3] Ninety-five percent of usual-type adenocarcinomas were HPV-positive, while 90% were p16-positive (Figure 2). All IECC HPV-associated mucinous carcinomas (mucinous NOS, mucinous intestinal, and invasive mucinous carcinomas with a resemblance to stratified mucin-producing intraepithelial lesion [iSMILE]) were HPV-positive, while only 69% were p16-positive. No gastric, endometrioid, serous or clear cell carcinomas were HPV-positive, although 33% of gastric-type and 17% of clear cell carcinomas were p16-positive. The ACD RNAscope® HPV HR18 probe set had superior sensitivity, specificity, and positive and negative predictive values (0.955, 0.968, 0.992, 0.833, respectively), compared to p16 (0.872, 0.632, 0.907, 0.545, respectively), in identifying HPVA usual and mucinous adenocarcinomas.

Markers associated with differentiation

Results are summarized in Table 3 and in Figures 3, 4, and 5. Expression of these markers is not related to HPV. Nearly all adenocarcinomas were CK7-positive, while CK20 expression was negative in all cases. At least 75% of all tumor types well-represented in the TMA were PAX8-positive with the notable exception of iSMILEs, which only displayed 14.3% positivity. Vimentin was positive in 13% of usual-type, 13% of iSMILE, 7.4% of gastric-type and 14% of clear cell carcinomas. All HPV-associated mucinous carcinomas aside from iSMILEs (mucinous NOS and intestinal-type), were Vimentin negative. PR was expressed in 20% of usual-type carcinoma and in small numbers of other tumor types, while ER was expressed in 5% of usual-type carcinomas. Sixty-five percent of ER-positive tumors were PR-positive and 30% of PR-positive tumors were ER-positive. Only 3 endocervical adenocarcinomas were classified as endometrioid. Of these, ER and PR were expressed in only 1 example each, 2 were PAX8 positive and all expressed CK7.

A summary of results for markers that have been proposed to be characteristically expressed in specific tumor types (i.e. HNF-1beta, napsin A, HIK1083, MUC6, CA-IX, GATA3 and TTF-1), and possibly useful in differential diagnosis, as well as those related to intestinal differentiation (CDX2 and SATB2), can be found in Table 4. HNF-1beta did not have the anticipated discriminatory power to distinguish clear cell carcinoma from other tumor types, nor did MUC6 or CA-IX for gastric-type carcinoma. In addition to demonstrating variable expression in clear cell carcinoma, HNF1beta was ubiquitously expressed in gastric-type and HPV-associated intestinal mucinous adenocarcinomas and in the majority of usual-type carcinomas. Napsin A marked equivalent numbers of clear cell carcinomas (42.9%), but was also expressed in approximately one-quarter of usual and gastric-type carcinomas, all intestinal-type HPVAs, and small numbers of tumors in other categories. Fifty-seven percent of clear cell carcinomas (4/7) would be regarded as “positive” had 1+ staining met criteria for a positive result. HIK1083 was expressed in 42% of gastric-type adenocarcinomas, but not in other tumor types. Sixty-eight percent of gastric-type carcinomas were positive for either MUC6 or CA-IX. However, MUC6 expression was also found in 25% of usual-type and almost 50% of mucinous adenocarcinomas, whether HPV-associated or not (i.e. HPVA mucinous adenocarcinomas and gastric-type adenocarcinomas). MUC6 expression was not

found in clear cell carcinoma, not an uncommon mimicker of gastric-type carcinomas. CA-IX was expressed in two-thirds of usual-type carcinomas and 80% of HPV-associated mucinous carcinomas (mucinous NOS and intestinal mucinous > iSMILE) but not in clear cell carcinomas. Given the frequent presence of goblet cells in both HPVA-intestinal mucinous adenocarcinoma and NHPVA gastric-type carcinoma,[3] we speculated that SATB2 and CDX2 might be used to detect lower intestinal differentiation; positive results would negate the usefulness of these markers in the differential diagnosis of metastatic intestinal adenocarcinomas, while negative results would provide indirect support for an endocervical adenocarcinoma containing goblet cells in the proper context. Overall, CDX2 expression was rare, found in only 1 of 4 HPV-associated mucinous carcinomas of intestinal-type, 2 of 25 gastric-type carcinomas, and in 3% of all usual-type carcinomas. SATB2 expression was found only in single examples of clear cell and endometrioid carcinomas. GATA3 positivity was identified in only 9.9% of usual-type adenocarcinomas, and in single examples of iSMILE and adenocarcinoma NOS. The single mesonephric carcinoma in the study was not adequately represented in the TMA used for GATA3 expression. TTF-1 was negative in all tumor types studied.

p63 and p40, markers of squamous differentiation, showed a discrepant rate of positivity as p63 was positive in only 3 cases (1 usual-type, 1 gastric-type and 1 iSMILE) while p40 positivity was encountered in 12% of usual-type adenocarcinomas, 29% of iSMILEs (Figure 3) and 43% of adenocarcinomas NOS. p40 was also present as a rim of positive cells at the periphery of the tumor cell nests in iSMILE (Table 5).

Markers of possible therapeutic importance

HER2 and androgen receptor (AR) results are presented in Table 6. HER2 overexpression was found in only 5 adenocarcinomas in the entire cohort. Most tumors were negative for both markers, with only 3.8% of the gastric-type (Figure 5) and 12.5% of the iSMILE-type positive for HER2. AR positivity was demonstrated in 5.9% of usual-type adenocarcinomas and in rare examples of other tumor types. Twenty-two percent of PR-positive tumors were AR-positive.

P53—a marker of possible prognostic significance

These results are presented in Table 7. Among HPVAs, aberrant p53 expression was found in only 3.6% of usual-type carcinomas (Figure 3), but was more commonly found in both HPVAs of mucinous type (Figure 4) and in NHPVAs, including 3 of 16 mucinous HPVAs (2 of them iSMILEs) and 52% of gastric-type carcinomas.

Discussion

Recent studies have demonstrated that, unlike cervical squamous cell carcinomas, approximately 15% of ECAs are HPV-unrelated (NHPVAs),[1,3,18]. HPVA ECAs, the most frequent variant being usual-type, have a better prognosis than NHPVA ECAs, of which the most frequent variant is gastric-type.[3,8] HPV status provides not only prognostic information, but also evidence regarding site of origin, as endometrial, ovarian and colorectal adenocarcinomas are HPV negative.[19–21]

To expand on this notion and assess the immunophenotype of ECA variants, with the goal of increasing diagnostic accuracy, we performed an extensive IHC evaluation of up to 297 ECAs as classified by IECC and represented in TMAs.

Our results for PAX8, Vimentin and ER/PR are broadly concurrent with those reported in the literature.[9,21–26] The majority of ECAs (all types) are PAX8-positive, although a significant minority, particularly iSMILE adenocarcinomas, are negative. Extensive PAX8 positivity should be useful in assigning a carcinoma of unknown origin to the gynecologic tract, provided that renal and thyroid carcinomas--which are highly unlikely to metastasize to the cervix--are excluded. Although most mucinous carcinomas, irrespective of HPV status, were negative for Vimentin, 12–14% of usual-type and clear cell carcinomas were at least focally positive for that marker. Up to 24% of usual-type adenocarcinomas showed at least focal PR expression, and 12% showed ER positivity, which could create difficulties in the differential diagnosis of these tumors and endometrial endometrioid carcinomas. Both HPV-ISH and p16 are far more robust discriminators when endometrial endometrioid carcinoma is a diagnostic consideration, although p16 can be overexpressed in up to 25% of endometrial endometrioid carcinomas, mostly FIGO grade 3.[27,28] As only 3 endometrioid carcinomas of endocervix were found in this large cohort, definite conclusions cannot be drawn about their immunophenotype. Our previous study,[3] summarized herein, reported that the ACD RNAscope® HPV HR18 probe set had superior sensitivity, specificity, and positive and negative predictive values, compared to p16, in identifying HPVA usual-type and mucinous adenocarcinomas of the cervix.

With the exception of HIK1083, which is specific but not highly sensitive for gastric-type adenocarcinoma in the current study, other markers of potential value in ECA subtyping (HNF-1beta, MUC6 and CA-IX) did not appear to be useful for this purpose. Approximately 60–70% of gastric-type adenocarcinomas were positive for either MUC6 or CA-IX, frequencies somewhat lower than that reported in the literature.[9,29] It was previously reported that MUC6 fails to distinguish gastric-type from other mucinous ECAs.[29] In this study, 40% of gastric-type carcinomas were HIK1083-positive, less than has been previously reported (75–100%),[11,29] although in one study gastric-type adenocarcinomas showed frequent focal or multifocal staining.[11] We, therefore, believe that the relatively low rates of positivity found in this study are likely related to the use of TMAs rather than whole sections. Unfortunately, HIK1083 is not commercially available outside of Japan to our knowledge, limiting its use in routine practice. However, given an ECA with obvious intracytoplasmic mucin, HPV-ISH efficiently separates HPVA from gastric-type carcinoma.

Among ovarian carcinomas, hepatocyte factor-1β (HNF-1beta) was initially found to be a sensitive and specific marker for clear cell carcinoma,[30,31] although subsequent studies reported diminished specificity in the setting of endometrial clear cell carcinomas.[32] Furthermore, HNF-1beta has also been reportedly expressed in more than 90% of gastric-type carcinomas[9] and our study confirmed this finding. In addition to its limited value in distinguishing between gastric-type and clear cell carcinoma, HNF-1b was expressed in up to 65% of usual-type carcinomas, further limiting its usefulness in differential diagnosis among the different subtypes of endocervical carcinomas.

Only 3 of 7 endocervical clear cell carcinomas were positive for HNF-1b in the current study. Fadare's group[30] reported that 73% of endometrial clear cell carcinomas overall express this marker; however, this percentage falls to 67.7% when 1–3+ scores (out of 12) were considered negative, as in the current study, and they fall as low as 47% when tumors with 4–7+ staining are counted as negative (a tumor with 6+ staining might show 26% positivity with strong intensity). Napsin A has recently been touted as a marker with superior performance characteristics compared to HNF-1beta,[33] despite its usually patchy, weak and granular cytoplasmic staining. Napsin A and HNF-1beta both marked 3 of 7 clear cell carcinomas from the current study, while 57% (4/7) would be regarded "napsin positive" had 1+ staining met criteria for a positive result. Compared to HNF-1beta, napsin A exhibited less frequent staining than HNF-1beta in other tumor types. In one study[33] 75% of 49 endometrial CCCs showed more than 1+ napsin A staining; in another study on the same topic,[34] 66.7% of endometrial clear cell carcinomas were positive, but fully 60% of the total were described as showing "focal" or "rare" staining. In one of the only studies of HNF-1beta and napsin A staining in endocervical clear cell carcinomas,[35] 6/7 cases were strongly HNF-1beta positive, but only 3 of 7 were strongly napsin A positive. These studies are difficult to compare because of the different antibody clones employed and differing methodologies. Nevertheless, a diagnosis of clear cell carcinoma is based on gold standard histological features, all of which were present in the 7 tumors studied herein. Our HNF-1beta and napsin A results for clear cell carcinomas remain within the same range as the results from some of the previously published studies, and might have appeared better had more endocervical clear cell carcinomas and whole sections been available for study.

Tp53 mutation (and aberrant p53 immunostaining) is reportedly significantly less frequent in HPVAs than in other carcinomas of the gynecologic tract,[36] most notably serous and serous-like carcinomas (carcinosarcomas and copy number-high endometrioid and clear cell carcinomas) of endometrium and ovary. The TCGA study of ECAs reported only 2 adenocarcinomas (1 endocervical and 1 endometrioid) with a *Tp53* mutation.[37] Previous studies have suggested a link between stage and *Tp53* mutation,[38] which makes sense if one assumes that most high-stage tumors were NHPVAs. A recent series reported aberrant p53 staining in 41% of gastric-type carcinomas,[9] while in the current study 51% of gastric-type carcinomas showed aberrant p53 staining. One of 7 clear cell carcinomas, another NHPVA, also showed p53 overexpression, which has been previously described.[35] In general, HPVAs were much less frequently p53-aberrant, although rates ranged from 3% in usual-type to 28% in HPVA mucinous carcinomas (rare intestinal-type HPVAs and iSMILEs), respectively. These results are similar to the findings of Park et al. that p53 was diffusely positive in almost half of gastric-type cases, whereas usual-type adenocarcinomas showed mostly negative staining, and other variants showed focal staining.[11] Although wild-type (physiological) p53 staining would be typical of usual-type carcinoma, aberrant staining would not distinguish between other types of ECAs.

This study, which has many of the characteristics of a population-based study, included only limited numbers of rare tumor types subjected to IHC. These included HPVA intestinal-type, mucinous NOS, and signet ring cell carcinomas, NHPVAs of endometrioid, serous and mesonephric types, and adenocarcinomas NOS. The immunophenotype of the 3 putative endocervical endometrioid carcinomas (all unassociated with endometriosis) was not typical

of endometrial endometrioid carcinoma, further undermining the validity of a diagnosis of endocervical endometrioid carcinoma in the absence of endometriosis, while the adenocarcinoma NOS category is heterogeneous, including both HPV-positive and negative adenocarcinomas. With respect to HPVA mucinous ECAs of which only as many as 7 mucinous NOS and intestinal-type adenocarcinomas were studied, iSMILE adenocarcinomas displayed some notable differences, such as more prevalent p40 expression and less prevalent PAX8, with possibly more frequent aberrant p53 staining. These data suggest that iSMILEs might diverge from other mucinous HPVAs, and could be categorized separately if clinical outcomes data support that conclusion.

Diagnostic Algorithms

Published reports regarding the immunophenotype of HPVA mucinous adenocarcinomas, “endocervical serous carcinomas,” mesonephric carcinomas and endometrial endometrioid carcinomas[39–59] can nevertheless be used in concert with the data presented here to construct diagnostic algorithms. For tumors with limited cytoplasmic mucin (Figure 5), HR-HPV ISH separates HPVA usual-type adenocarcinoma from endometrial endometrioid, clear cell and mesonephric carcinomas with 95% accuracy. Expression of ER, very rarely seen in mesonephric and clear cell carcinomas, separates endometrial endometrioid carcinomas from NHPVAs. If using only one steroid receptor marker, ER may outperform PR because the former marks 50% fewer usual-type carcinomas in this study. However, once usual-type carcinomas are excluded by HR-HPV ISH, there are no obvious advantages to one over the other. The very low rate of ER or PR expression in NHPVAs makes these markers important when exploring the differential diagnosis with endometrial endometrioid adenocarcinoma, where rates of ~90% have been reported in FIGO grades 1 and 2 endometrial endometrioid carcinomas.[32] GATA3 expression could then separate the remaining NHPVAs, mesonephric and clear cell carcinomas, as we report 0% positivity for GATA3 in clear cell carcinoma, while the limited published data about GATA3 expression in endocervical mesonephric carcinomas reveal that 96% of 24 cases are GATA3-positive.[60–62]

In mucinous adenocarcinomas (Figure 6), HPV-ISH again separates NHPVAs from mucinous carcinomas of HPVA type. ER can then be used to separate mucinous carcinoma of endometrium from gastric-type adenocarcinoma, with 75–90% expression in the former[63,64] and no expression in the latter. All of these immunohistochemical stains are easily interpreted, but distinguishing between an HPV-positive ECA with only rare nuclear or cytoplasmic signals using HR-HPV ISH and an NHPVA can on rare occasions cause difficulties. These were encountered in 3 of 232 cases. In each case, the questionable signal was compared to the negative control and found to be false positive. p16 can also be used to score challenging cases, but, as detailed in this and other manuscripts, rare HPVAs are p16-negative and many NHPVAs are positive.

The algorithms presented here are meant to provide a diagnostic guide for pathologists lacking close familiarity with usual patterns of HPVAs (i.e. invasive SMILE or intestinal-type mucinous HPVAs) and NHPVAs (i.e. gastric-type carcinoma, particularly), and cytoarchitectural differences between usual-type and mucinous HPVAs on the one hand and undifferentiated endometrioid carcinomas of endometrium on the other. Following the

published IECC guidelines,[3] which are based on evaluation of H&E slides only, a pathologist should be able to confidently distinguish between HPVAs and NHPVAs in most cases. Ancillary testing, such as mRNA-based HR-HPV ISH, or the less sensitive and specific p16, would then be used only in diagnostically difficult cases.

We therefore propose diagnostic immunohistochemical algorithms that can be used to distinguish histologic subtypes of ECAs classified by the IECC, as well as endometrial endometrioid carcinomas. These algorithms will improve diagnostic concordance and differentiation between sites of origin.

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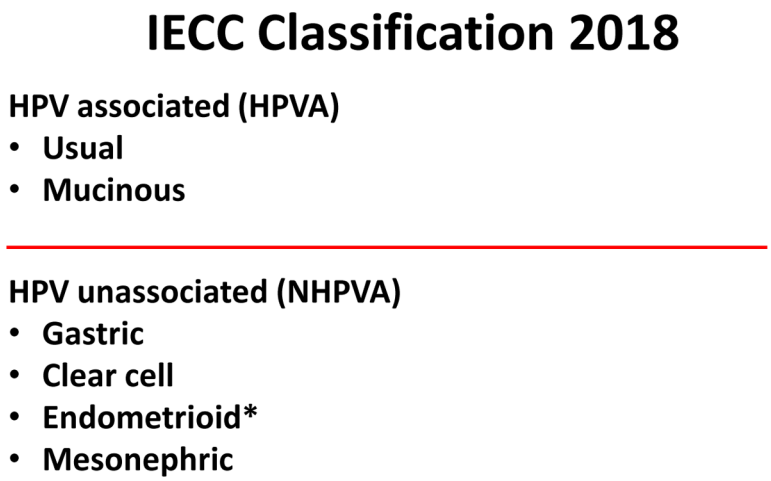

Figure 1.
IECC Classification 2018.
HPVA mucinous carcinomas include mucinous ECA, not otherwise specified, mucinous ECA of intestinal type, mucinous ECA of signet ring-cell type and "invasive SMILE".[65]
*Endometrioid carcinoma is very rarely encountered as an endocervical primary tumor. Little is known about its derivation, clinical correlates, and biological properties.[3]

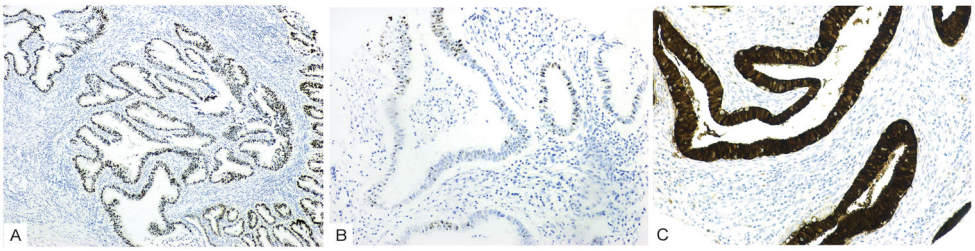

Figure 2.

Usual-type HPV-associated endocervical adenocarcinoma (Usual-type HPVA). Diffuse signals with HR-HPV ISH (**A**, low magnification; **B**, high magnification) and p16 (**C**, block-like staining).

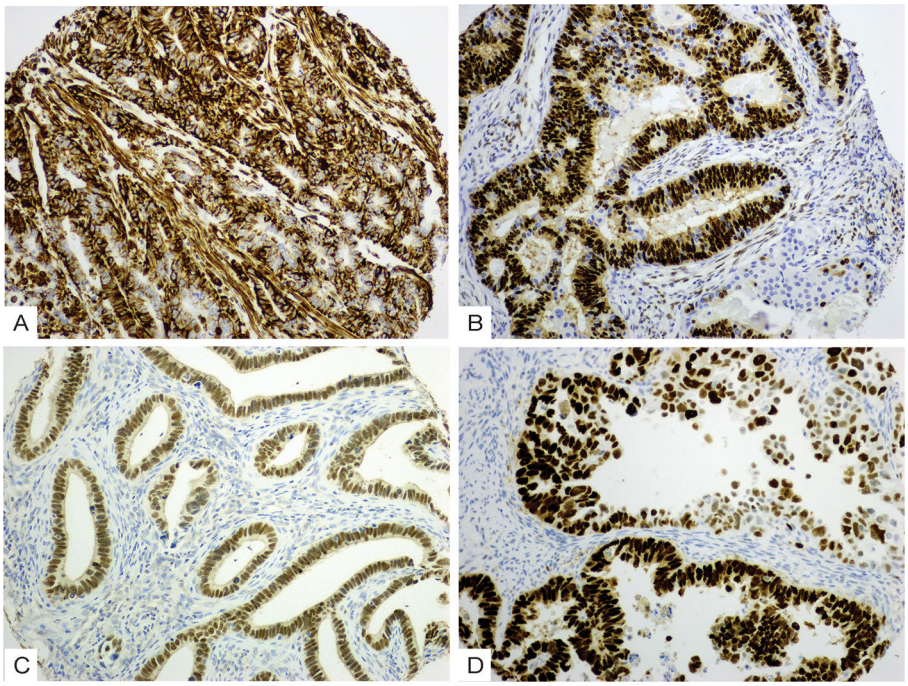

Figure 3.

Usual-type HPV-associated endocervical adenocarcinoma (Usual-type HPVA) with unexpected results. Vimentin expression (**A**); Progesterone receptor (PR) expression (**B**); Hepatocyte nuclear factor 1beta (**C**); p53 overexpression (**D**). Although uncommon, expression of vimentin and PR can be encountered. Hepatocyte nuclear factor 1beta expression is common, but aberrant staining with p53 is rare.

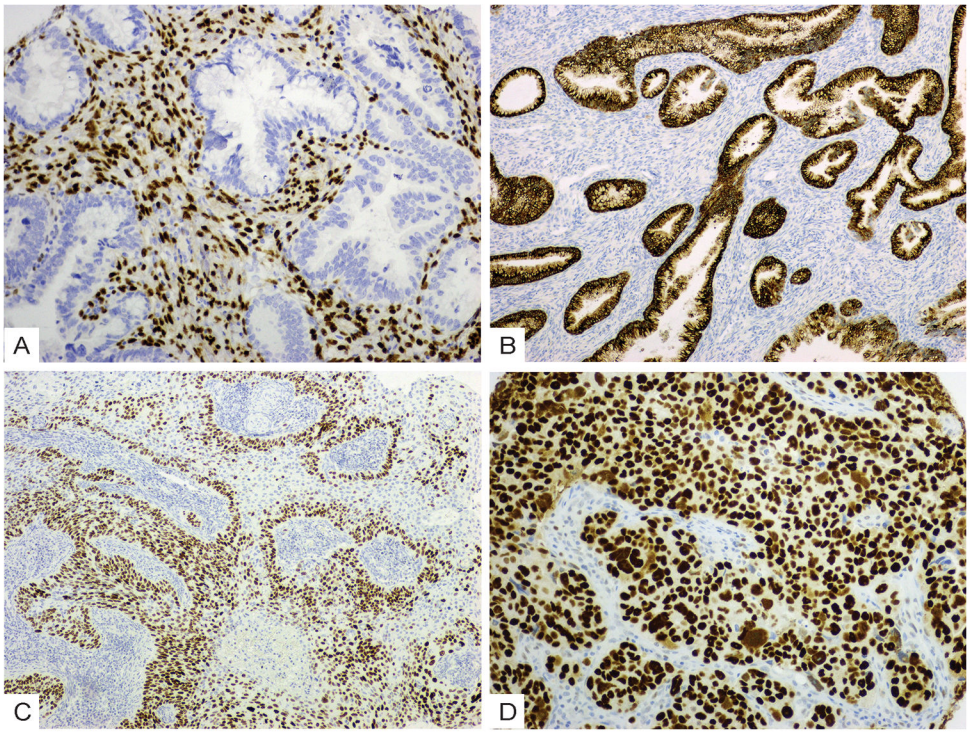

Figure 4.

Mucinous HPV-associated endocervical adenocarcinoma (Mucinous HPVA), including invasive carcinoma resembling stratified mucin-producing intraepithelial lesion (iSMILE). Lack of PR expression in mucinous HPVA (**A**); MUC6 expression in mucinous HPVA (**B**); p63 expression in iSMILE (**C**); p53 overexpression in iSMILE (**D**). Most mucinous HPVAs are PR-negative, but MUC6 expression is common. Unlike other HPVAs, iSMILEs can show p63 expression that is usually accentuated in the basaloid peripheral palisade. Like other mucinous HPVAs, aberrant p53 expression is not uncommon in iSMILEs. Aberrant p53 expression, however, is much more commonly observed in gastric-type carcinomas (mucinous NHPVA) and, to a lesser extent, clear cell carcinoma.

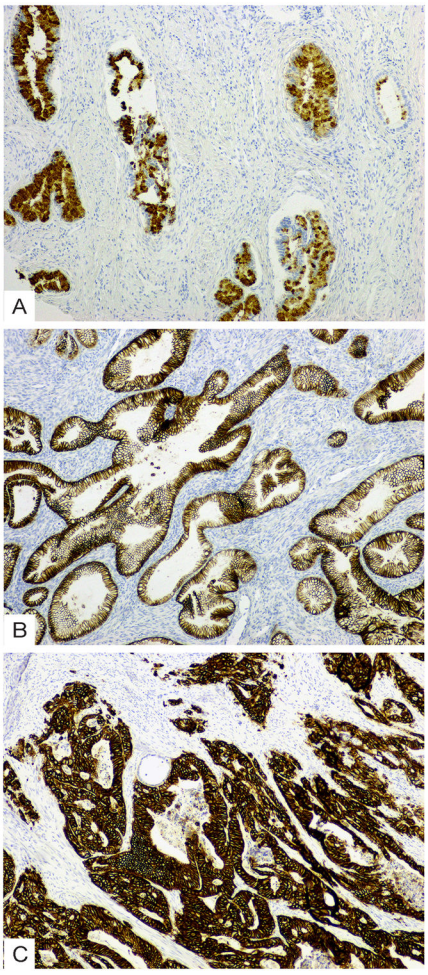

Figure 5.
Gastric-type carcinoma (Mucinous NHPVA). HIK1083 expression (**A**); CA-IX expression (**B**); and HER2 overexpression (**C**)

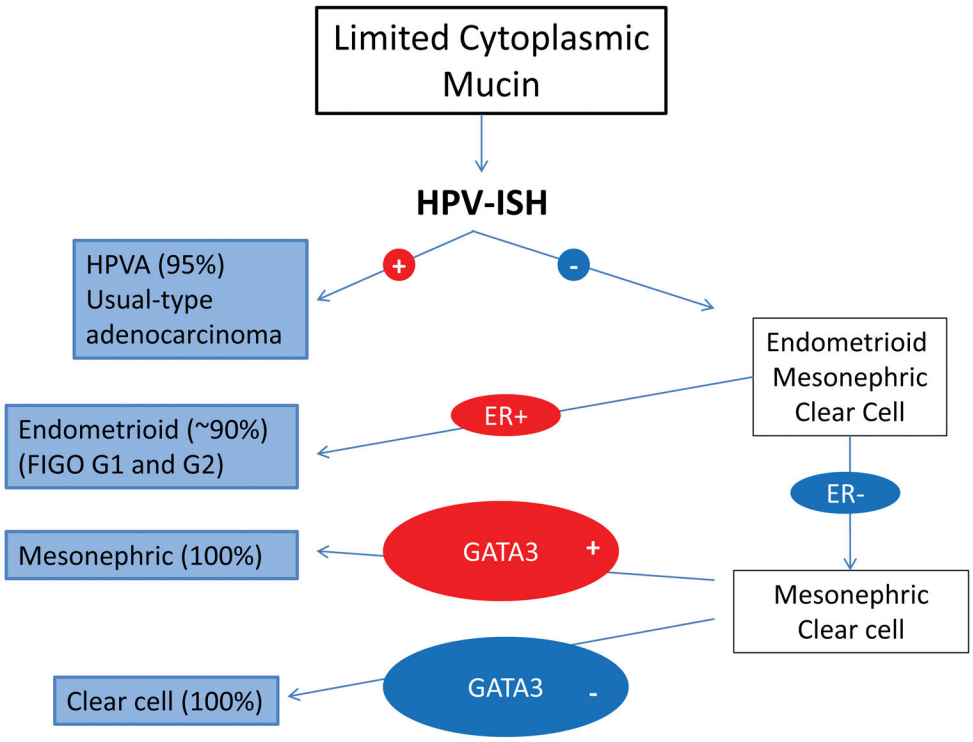

Figure 6.
Immunohistochemical algorithm for ECAs with limited cytoplasmic mucin.

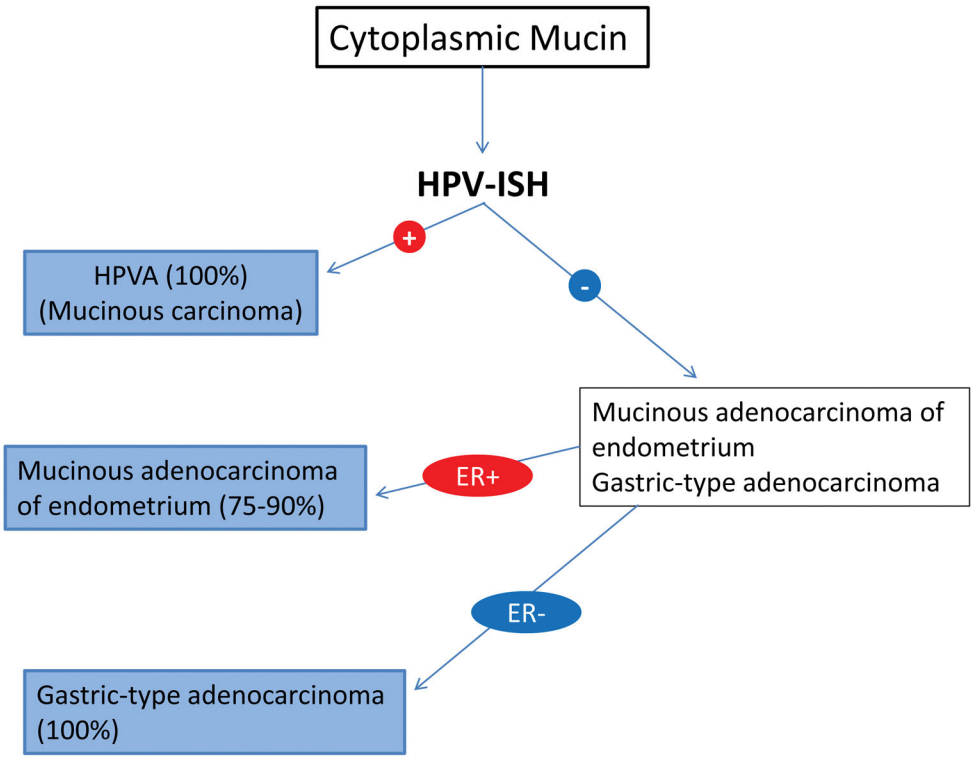

Figure 7.
Immunohistochemical algorithm for ECAs containing obvious cytoplasmic mucin.

Table 1

IECC Criteria

		Tumor subtype		Morphologic features
HPVA	Apical mitotic figures and apoptotic bodies easily appreciable at scanning magnification	**USUAL**		0–50% of cells with appreciable intracytoplasmic mucin, +/− benign squamous differentiation
		HPVA-MUCINOUS	MUCINOUS NOS	≥50% of cells with intracytoplasmic mucin in a background of usual-type
			MUCINOUS INTESTINAL	≥50% of cells with goblet morphology in a background of usual-type
			MUCINOUS SIGNET-RING	≥50% of tumor cells with signet-ring morphology in a background of usual-type
			INVASIVE SMILE	Invasive nests of stratified columnar cells with peripheral palisading and variable amounts of intracytoplasmic mucin
		VILLOGLANDULAR		Usual-type cytomorphology with exophytic long slender papillae
ADENOCARCINOMA NOS		Any tumor that could not be classified by IECC		
NHPVA	Absence of easily-identifiable mitotic activity and apoptotic bodies at scanning magnification	**GASTRIC**		Cells with abundant clear, foamy or pale eosinophilic cytoplasm, distinct cytoplasmic borders, generally low nuclear-cytoplasmic ratios and irregular basally-located nuclei, limited or no HPVA-like features
		CLEAR CELL		Solid, papillary and/or tubulocystic architecture with polygonal cells and highly atypical but uniform nuclei
		ENDOMETRIOID		Endometrioid morphology with “confirmatory features” (at least focally identified low-grade endometrioid glands lined by columnar cells, with pseudostratified nuclei demonstrating no more than moderate atypia, +/− squamous differentiation and/or endometriosis)
		SEROUS		Papillary and/or micropapillary architecture with cells showing diffusely distributed, highly atypical nuclei in stratified and pseudostratified cells
		MESONEPHRIC		Admixture of growth patterns (ductal, tubular, papillary, cord-like and others) as well as intraluminal eosinophilic colloid-like material resembling mesonephric remnants

Table 2

Immunohistochemical Antibodies

Antibody	CLONE	VENDOR	Instrument (dilution)
Vimentin	V9	Roche	Roche Discovery XT
p53	D07	Roche	Roche Benchmark Ultra
p16	E6H4	Roche	Roche Benchmark Ultra
PAX8	Poly	Protein Tech	Roche Benchmark Ultra
AR	Poly	Santa Cruz Biotechnology	Roche Discovery XT
PR	1E2	Roche	Roche Discovery XT
HER2	4B5	Roche	Roche Discovery XT
HIK1083	HIK1083	Kanto	Manual (1/20)
MUC 6	CLH5	Novocastra	Manual (1/200)
CA IX	Poly	Novus	Roche Benchmark Ultra
SATB2	EP281	Cell Marque	Roche Benchmark Ultra
HNF1beta	CLO374	Sigma	Leica Bond III
Napsin A	Poly	Nichirei Biosciences	Discovery Ultra Ventana
CK7	OV-TV12/30	DAKO	Roche Benchmark Ultra
CK20	KS20.8	DAKO	Roche Benchmark Ultra
CDX2	CDX2-88	Biogenex	Roche Benchmark Ultra
P63	4A4	Roche	Roche Benchmark Ultra
P40	BC28	Biocare	Roche Benchmark Ultra

Table 3

Markers associated with differentiation and site of origin

SUBTYPE	N TMA	VIM % (n)	PR % (n)*	ER % (n)**	PAX 8 % (n) ***	CK7 % (n)#	CK20 % (n) ##	TTF 1 % (n)
USUAL	140–173	12.7 (22)	20.0 (34)	5 (7)	64.5 (98)	93.0 (144)	0	0
GASTRIC	25–27	7.4 (2)	12.0 (3)	0	80.0 (20)	96.0 (24)	0	0
MUCINOUS NOS	3–4	0	0	0	50.0 (1)	100.0 (2)	0	0
INTESTINAL	4	0	25.0 (1)	25 (1)	75.0 (3)	100.0 (4)	0	0
SIGNET RING	-	-	-	-	-	-	-	0
CLEAR CELL	7	14.3(1)	0	14.2 (1)	85.7 (6)	100.0 (7)	0	0
iSMILE	7–8	12.5 (1)	25.0 (2)	14.2 (1)	14.3(1)	100.0 (7)	0	0
ADENO NOS	5–7	0	40.0 (2)	33.3 (2)	42.9 (3)	100.0 (7)	0	0
ENDOMETRIOID	3	0	33.3 (1)	33.3 (1)	66.7 (2)	100.0 (3)	0	0
VILLOGLANDULAR	1–2	0	0	0	100.0 (2)	100.0 (2)	0	0
SEROUS	1–2	100.0 (1)	-	100 (1)	100.0 (2)	100.0 (2)	0	0
MESONEPHRIC	1	0	0	-	-	-	0	-

N TMA: range of cases represented in the TMA for each marker

n=number of positive cases (3–4+ staining where applicable)

*(PR): 5 usual-type HPVAs showed 1+ staining and 2 showed 2+ staining; 1 clear cell carcinoma showed 2+ staining

**(ER): 4 usual-type HPVAs showed 1+ staining and 5 showed 2+ staining

***(PAX8): 11 usual-type cases showed 1+ staining and 5 showed 2+ staining; 1 case each of mucinous NOS HPVA and gastric-type NHPVA showed 1+ staining; 1 case each of gastric-type NHPVA and iSMILE showed 2+ staining

#(CK7): 2 usual-type HPVAs showed 1+ staining

##(CK20): 1 case each of usual-type and gastric-type NHPVA showed 1+ staining

Table 4

Markers associated with mucinous and clear cell differentiation

SUBTYPE	N TMA	MUC 6 % (n)	HIK 1083 % (n)	CA IX % (n)	CDX2 % (n)	SATB2 % (n)	HNF1b % (n) *	Napsin A%(n) **
USUAL	120–157	24.4 (38)	0.6 (1)	67.8 (103)	3.3 (5)	0.7 (1)	64.5 (98)	22.4 (28)
GASTRIC	24–25	45.8(11)	41.7 (10)	60.0 (15)	8.0 (2)	0	92.0 (23)	25 (6)
MUCINOUS NOS	2–3	0	0	100 (2)	0	0	0	0
INTESTINAL	3–4	75.0 (3)	25.0 (1)	75.0 (3)	25.0 (1)	0	100.0 (4)	66.6 (2)
SIGNET RING	-	-	-	-	-	-	-	-
CLEAR CELL	7	0	0	0	0	14.3 (1)	42.9 (3)	42.9 (3)
iSMILE	7	57.2 (4)	0	57.2 (4)	0	0	14.3 (1)	14.3 (1)
ADENO NOS	6–7	0	0	16.7 (1)	0	0	71.4 (5)	16.7 (1)
ENDOMETRIOID	2–3	33.3 (1)	0	50.0 (1)	0	33.3 (1)	0	0
VILLOGLANDULAR	1–2	50.0 (1)	0	50.0 (1)	0	0	50.0 (1)	0
SEROUS	1–2	50.0 (1)	0	0	0	0	50.0 (1)	50.0 (1)
MESONEPHRIC	-	-	-	-	-	-	-	-

N TMA: range of cases represented in the TMA for each marker

n=number of positive cases (3–4+ staining where applicable)

*(HNF-1b): 1 villoglandular and 1 usual-type HPVA showed 1+ staining

**(Napsin A): 2 usual-type, 1 clear cell and 1 adenocarcinoma NOS showed 1+ staining

Table 5

Markers associated with squamous differentiation

SUBTYPE	N TMA	p63 % (n)	p40 % (n)	GATA 3 % (n)
USUAL	151–169	0.6 (1)	7.9 (12)	9.9 (15)
GASTRIC	25–26	4.2 (1)	4.0 (1)	0
MUCINOUS NOS	2–4	0	0	0
INTESTINAL	4	0	0	0
SIGNET RING	-	-	-	-
CLEAR CELL	7	0	0	0
iSMILE	7	25.0 (1)	28.6 (2)	14.3 (1)
ADENO NOS	6–7	0	42.9 (3)	14.3 (1)
ENDOMETRIOID	3	0	0	0
VILLOGLANDULAR	1	-	0	0
SEROUS	2	0	0	0
MESONEPHRIC	1	0	-	-

N TMA: range of cases represented in the TMA for each marker

n=number of positive cases

Table 6

Markers associated with therapeutic prediction

SUBTYPE	N TMA	HER 2 % (n)	AR % (n)
USUAL	166–7	1.8(3)	1.8 (3)
GASTRIC	26	3.8(1)	0
MUCINOUS NOS	4–5	0	0
INTESTINAL	3	0	0
SIGNET RING	-	-	-
CLEAR CELL	7	0	14.3 (1)
iSMILE	8	12.5 (1)	0
ADENO NOS	6–7	0	28.6 (2)
ENDOMETRIOID	3	0	0
VILLOGLANDULAR	1–2	0	0
SEROUS	1	-	100.0 (1)
MESONEPHRIC	1	0	0

N TMA: range of cases represented in the TMA for each marker

n=number of positive cases

Table 7

p53 analysis

SUBTYPE	N TMA	p53 % (n)
USUAL	168	3.6 (6)
GASTRIC	27	51.9 (14)
MUCINOUS NOS	4	0
INTESTINAL	4	25.0 (1)
SIGNET RING	-	-
CLEAR CELL	7	14.3 (1)
iSMILE	8	28.6 (2)
ADENO NOS	6	16.7 (1)
ENDOMETRIOID	3	33.3 (1)
VILLOGLANDULAR	2	50.0 (1)
SEROUS	2	0
MESONEPHRIC	1	0

N TMA: range of cases represented in the TMA for each marker

n=number of cases with aberrant staining