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# Immunohistochemical p16 overexpression and Rb loss correlate with high-risk human papillomavirus infection in endocervical adenocarcinomas

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## Immunohistochemical p16 overexpression and Rb loss correlate with high-risk human papillomavirus infection in endocervical adenocarcinomas

*Aims*: p16 is a sensitive surrogate marker for transcriptionally active high-risk human papillomavirus (HR-HPV) infection in endocervical adenocarcinoma (ECA); however, its specificity is not perfect.

*Methods and results*: We examined p16 and Rb expressions by immunohistochemistry (IHC) and the transcriptionally active HR-HPV infection by mRNA *in-situ* hybridisation (ISH) with histological review in 108 ECA cases. Thirteen adenocarcinomas of endometrial or equivocal origin (six endometrioid and seven serous carcinomas) were compared as the control group. HR-HPV was detected in 83 of 108 ECA cases (77%), including five HPV-associated adenocarcinomas *in situ* and 78 invasive HPV-associated adenocarcinomas. All 83 HPV-positive cases showed consistent morphology, p16 positivity and partial loss pattern of Rb. Among the 25 cases of HPV-independent adenocarcinoma, four (16%) were positive for p16, and of these four cases, three of 14 (21%) were gastric type adenocarcinomas and one of 10 (10%) was a clear cell type adenocarcinoma. All 25 HPV-independent adenocarcinomas showed preserved expression of Rb irrespective of the p16 status. Similarly, all 13 cases of the control group were negative for HR-HPV with preserved expression of Rb, even though six of 13 (46%) cases were positive for p16. Compared with p16 alone, the combination of p16 overexpression and Rb partial loss pattern showed equally excellent sensitivity (each 100%) and improved specificity (100 versus 73.6%) and positive predictive values (100 versus 89.2%) in the ECA and control groups. Furthermore, HR-HPV infection correlated with better prognosis among invasive ECAs.

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**Abbreviations:** AIS, adenocarcinoma *in situ*; CDX2, Caudal related homeobox gene type2; ECA, endocervical adenocarcinoma; ER, oestrogen receptor; FFPE, formalin-fixed paraffin-embedded; FIGO, International Federationof Gynaecology and Obstetrics; GATA3, GATA binding protein 3; HNF1β, hepatocyte nuclear factor1-beta; HR-HPV, high-risk human papillomavirus; IHC, immunohistochemistry; ISH, *in-situ* hybridization; NOS, not otherwise specified; OS, overall survival; PgR, progesterone receptor; SCC, squamous cell carcinoma; SMILE, stratified mucin-producing intraepithelial lesion; WHO, World Health Organisation.

*Conclusions*: The results suggest that the combined use of p16 and Rb IHC could be a reliable method to predict HR-HPV infection in primary ECAs and mimics. This finding may contribute to prognostic prediction and therapeutic strategy.

Keywords: endocervical adenocarcinoma, human papillomavirus, p16, Rb, uterus

## Introduction

High-risk human papillmavirus (HR-HPV) infection is closely associated with the occurrence of epithelial neoplasms of the uterine cervix, including squamous intraepithelial neoplasia, squamous cell carcinoma (SCC), adenocarcinoma in situ (AIS) and invasive adenocarcinoma.[1–3] The relative and real incidence of endocervical adenocarcinomas (ECAs) has been increasing worldwide, and up to 20–25% of all invasive cervical tumours belong to this category.[4]

The histological classification of ECAs had been based on invasiveness, cellular morphology and mucin, production rather than on HPV infection, in the former fourth World Health Organisation (WHO) classification of tumours of the female reproductive organs. For example, intestinal type (HPV-positive) and gastric type (HPV-negative) adenocarcinomas had been placed into the category of 'mucinous' carcinomas.[5] However, the current fifth WHO classification has shifted the priority to the presence or absence of HR-HPV infection and set up the categories of 'HPV-associated' and 'HPV-independent' adenocarcinomas.[2] HPV-associated AIS can show morphological variations such as conventional type and mucinous type; the latter includes stratified mucin-producing intraepithelial lesion (SMILE). HPV-associated adenocarcinoma – an invasive cancer – is roughly classified into usual and mucinous types, and histological patterns are further assigned for each type; e.g. usual type (conventional, micropapillary pattern and villoglandular variant) and mucinous type [not otherwise specified (NOS), intestinal adenocarcinoma, signet-ring cell adenocarcinoma and stratified mucin-producing carcinoma]. HPV-independent AIS is rare. HPV-independent adenocarcinomas are further subclassified into gastric, clear cell, mesonephric and endometrioid types. This reclassification of ECAs is based on the recent advances in the knowledge on epidemiology, clinicopathological features and biological behaviours of HPV-associated and independent ECAs.[4] For example, gastric type HPV-independent adenocarcinomas have been reported to have worse prognosis when compared with HPV-associated adenocarcinomas.[6–8] HPV-independent endometrioid adenocarcinomas are now considered extremely rare, accounting for no more than 1% of all primary ECAs.[4] This category should be distinguished from cervical invasion of endometrioid carcinoma of the endometrium origin.[2,9] According to the recent consensus, serous carcinoma detected in the cervix represents metastasis or direct extension from adnexal or endometrial serous carcinoma; thus, the entity of 'serous carcinoma' is not listed as a primary tumour of the uterine cervix in the present fifth WHO classification.[2]

p16 immunohistochemistry (IHC) is an effective (but flawed) indirect test for HR-HPV infection. Approximately 95% of HPV-associated carcinomas showed p16 overexpression with diffuse block positivity or every-cell staining pattern.[4] p16 is highly sensitive for HR-HPV infection; however, it is not an entirely specific surrogate marker. That is, a subset of HPV-independent adenocarcinomas can be diffusely positive for p16.[3,10–12] In addition, because serous carcinoma (endometrial or adnexal origin), including that invading the cervix, often overexpresses p16, p16-positivity should not readily diagnose HPV-associated adenocarcinoma of the cervix.[2,13,14]

At the molecular level, in HPV-infected cells the HPV E7 protein binds to the Rb protein and induces not only p16 protein overexpression but also Rb protein degradation through the ubiquitin–proteasome pathway.[15] Previously, we found that HR-HPV infection was typically associated with the partial loss (mosaic) pattern of Rb expression by IHC, and we proposed that the combination of characteristic expression patterns of p16 and Rb could reliably predict HR-HPV infection in head and neck SCCs in the sinonasal tact,[16] oropharynx,[17] conjunctiva and lacrimal sac.[18] Conversely, *RB1* gene alteration (mutation and/or loss) can lead to the loss of Rb protein expression and overexpression of p16, and this phenomenon is frequently observed in neuroendocrine carcinoma of the lungs and pancreas.[19–21] The exact molecular mechanism underlying the diffuse expression of p16 in HPV-independent adenocarcinoma of the uterine cervix and serous carcinoma of the female genital tract is unclear. According to a previous

report and public database, *RB1* gene alteration is less common in these carcinomas.[22,23] A previous study has reported that histone modification with activation of lysine demethylase 6B and demethylation of histone 3 lysine 27 might be responsible for p16 overexpression in serous carcinoma of the uterine corpus.[24]

This study aimed to determine the potential benefits of the combined use of p16-IHC and Rb-IHC to predict HR-HPV infection in ECAs and evaluate the prognostic significance of HR-HPV infection in ECAs.

## Materials and methods

### CASE SELECTION

Biopsy specimens and surgically resected specimens of formalin-fixed paraffin-embedded (FFPE) tissues were retrospectively collected from 108 ECAs, which included five cases of HPV-associated AIS, 78 cases of HPV-associated adenocarcinoma and 25 cases of HPV-independent adenocarcinoma (Table 1). HPV-associated and HPV-independent adenocarcinomas were defined as invasive cancers according to the fifth WHO classification.[2] In addition, 13 patients with adenocarcinomas of endometrial or equivocal origin were examined as the control group. Here, uterine cancer of equivocal origin represents a tumour involving the boundary of upper endocervix and lower endometrium. All 121 patients were histopathologically diagnosed based on biopsy findings and subsequently underwent surgery or received radiation at Kyushu University between 1998 and 2022. Patients' clinical data were obtained from their medical records. Invasive ECAs were staged using the 2018 International Federation of Gynaecology and Obstetrics (FIGO) staging system. For old cases (1998–2017), the evidence was re-evaluated according to the new 2018 FIGO staging system for uterine cervical adenocarcinomas (Supporting information, Table S1). The Institutional Review Board at Kyushu University approved this study (permission code: 22050-00).

### HISTOPATHOLOGICAL EVALUATION

Two investigators (N.Y. and H.Y.) independently reviewed and diagnosed the patients according to the fifth edition of the WHO classification of female genital tumours.[2] HPV-associated AISs were subclassified into conventional and mucinous types, including SMILE. HPV-associated adenocarcinomas were classified into usual and mucinous types, and the predominant histological pattern was also recorded for usual type (conventional, micropapillary pattern and villoglandular variant) and mucinous type (NOS, intestinal adenocarcinoma, signet-ring cell adenocarcinoma and stratified mucin-producing carcinoma).[2] HPV-independent adenocarcinomas were subclassified into gastric type, clear cell type, mesonephric type, endometrioid adenocarcinoma and NOS. In our series, no patients had HPV-independent AIS and adenocarcinoma, mesonephric type. Endometrioid carcinomas from the uterine corpus or lower uterine segment were carefully excluded. Consequently, in our series, endometrioid carcinomas of absolutely endocervical origin were not recorded. Instead, 13 cases of uterine cancer of endometrial or equivocal origin (six endometrioid carcinomas and seven serous carcinomas) were used as the control group.

The above classification was made based on the morphological features and p16 IHC results. If necessary, ancillary IHC markers were performed, including p53 (clone DO-7, dilution 1/500; Novocastra, Newcastle upon Tyne, UK), oestrogen receptor (ER; clone EP1, prediluted; Dako, Glostrup, Denmark), progesterone receptor (PgR; clone PgR636, pre-diluted; Dako), hepatocyte nuclear factor 1-beta (HNF1β; polyclonal, dilution 1/100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HIK1083 (clone HIK1083, dilution 1/20; Kanto Kagaku, Tokyo, Japan), Caudal related homeobox gene type2 (CDX2) (clone EPR2764Y, pre-diluted; NICHIREI BIOSCIENCES INC., Tokyo, Japan), and GATA binding protein 3 (GATA3; clone L50-823, dilution 1/100; Cell Marque, Rocklin, CA, USA).

### P16 AND RB IHC

For the IHC, 4-μm-thick FFPE tissue sections and primary monoclonal antibodies against p16 (E6H4, prediluted, CIN histology kit; Roche, Heidelberg, Germany) and Rb (G3-245, dilution 1/50; BD Pharmingen, Franklin Lakes, NJ, USA) were used. The IHC methods were as described previously.[16,17] The p16 protein expression was considered positive (protein overexpression) if nearly all tumour cells showed strong and diffuse nuclear and cytoplasmic staining ('block' positivity).[25] A previously reported scoring system was utilised to categorise Rb-IHC expression as complete loss, preserved expression or partial loss, as follows[16–18]: for complete loss, Rb expression was present in < 10% (disappeared in > 90%) of the tumour cells; for preserved expression, Rb expression was present in > 90% of the tumour cells; and for partial loss, Rb expression was spotty (mosaic),

**Table 1.** Histological types, HPV infection and p16/Rb expressions in uterine cervical adenocarcinomas

| Histology | *n* = 121 | HR-HPV ISH, *n* (%) Positive | p16 IHC, *n* (%) Positive | Rb IHC, *n* (%) | | |
|---|---|---|---|---|---|---|
| | | | | Partial loss | Complete loss | Preserved |
| Study group (cervical origin) | 108 | 83 (77) | 87 (80) | 83 (77) | 0 (0) | 25 (23) |
| HPV-associated adenocarcinoma in situ | 5 | 5 (100) | 5 (100) | 5 (100) | 0 (0) | 0 (0) |
| Conventional adenocarcinoma in situ | 3 | 3 (100) | 3 (100) | 3(100) | 0 (0) | 0 (0) |
| Stratified mucin-producing intraepithelial lesion | 2 | 2 (100) | 2 (100) | 2 (100) | 0 (0) | 0 (0) |
| HPV-associated adenocarcinoma | 78 | 78 (100) | 78 (100) | 78 (100) | 0 (0) | 0 (0) |
| Usual type | 67 | 67 (100) | 67 (100) | 67 (100) | 0 (0) | 0 (0) |
| Mucinous type | 11 | 11 (100) | 11 (100) | 11 (100) | 0 (0) | 0 (0) |
| HPV-independent adenocarcinoma | 25 | 0 (0) | 4 (16) | 0 (0) | 0 (0) | 25 (100) |
| Gastric type | 14 | 0 (0) | 3 (21) | 0 (0) | 0 (0) | 14 (100) |
| Clear cell type | 10 | 0 (0) | 1 (10) | 0 (0) | 0 (0) | 10 (100) |
| Mesonephric type | 0 | – | – | – | – | – |
| Endometrioid adenocarcinoma | 0 | – | – | – | – | – |
| Not otherwise specified | 1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (100) |
| Control group (endometrial or equivocal origin) | 13 | 0 (0) | 6 (46) | 0 (0) | 0 (0) | 13 (100) |
| Endometrioid carcinoma | 6 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 6 (100) |
| Serous carcinoma | 7 | 0 (0) | 6 (86) | 0 (0) | 0 (0) | 7 (100) |

HPV, human papillomavirus; HR, high risk; IHC, immunohistochemical staining; ISH, *in-situ* hybridisation.

present in ≥ 10 to ≤ 90% of the tumour cells. For evaluation of Rb, we searched the most prevalent area (hot-spot) of immunoreactive tumour cells at low-power magnification (×10 ocular and ×4 objective), and then scored the percentages of Rb-expressing cells in one field at higher-power magnification (×10 ocular and ×20 objective).[17] Only nuclear staining was evaluated for Rb expression. Intratumoural endothelial cells were used as an internal positive control, and positive staining for Rb in those cells were confirmed to avoid false negativity.[17] Two pathologists (N.Y. and H.Y.) independently evaluated the Rb expression patterns without information regarding the p16 IHC and HPV ISH results.

### ISH FOR HIGH-RISK HPV MRNA

*In-situ* hybridisation (ISH) was performed to detect HPV RNA using an E6/E7 mRNA probe set (Advanced Cell Diagnostics, Newark, CA, USA) for 18 HR-HPV types (types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) with an RNA scope 2.5HD detection kit (Advanced Cell Diagnostics), according to the manufacturer's protocol. The presence of any nuclear or cytoplasmic dots was considered positive.[26] As a positive control for HPV-ISH, oropharyngeal SCC specimens were used.[16]

### STATISTICAL ANALYSIS

Statistical analysis was performed using Fisher's exact test for categorical variables. Univariate and multivariate regression analyses with Cox proportional hazards model were conducted to identify prognostic factors. A Kaplan–Meier analysis and the log-rank test were used to derive and compare survival curves. Overall survival (OS) was defined as the time from the first day of therapy until death or to the last follow-up date for censored cases. Analyses were performed with JMP16 (SAS Institute, Cary, NC, USA). *P*-values of $< 0.05$ were considered significant.

## Results

### CLINICAL FINDINGS

The clinicopathological findings of the 108 ECAs are summarised in Supporting information, Table S1. The ages ranged from 28 to 83 (median = 45) years, and 68% of the patients were diagnosed with stage I. The OS ranged from 1 to 245 (median = 30) months.

### HPV INFECTION AND P16/RB EXPRESSION STATUS

As shown in Table 1, among the 108 primary ECAs, HR-HPV mRNA ISH was positive in 83 (77%) cases and negative in 25 (23%). The five cases of HPV-associated AIS were subclassified into conventional AIS ($n$ = three) and SMILE ($n$ = two; Supporting information, Figure S1, Table 1). The 78 cases of HPV-associated adenocarcinomas were subclassified into usual ($n$ = 67) and mucinous ($n$ = 11) types (Figure 1, Supporting information, Figure S2 and Table 1). The 25 HPV-independent adenocarcinomas were subclassified into gastric type ($n$ = 14), clear cell type ($n$ = 10) and NOS ($n$ = one; Figures 2 and 3 and Table 1).

Among 108 ECAs, p16 was positive in 87 (80%), and Rb expression pattern was divided into partial loss ($n$ = 83; 77%) and preserved ($n$ = 25; 23%) patterns. All HPV-associated AISs and adenocarcinomas ($n$ = 83; 100%) showed p16 positivity and partial loss pattern of Rb (Figures 1 and 4). In HPV-independent adenocarcinomas, 21 of 25 cases (84%) were negative for p16, and the remaining four (16%) were diffusely positive for p16 (Figures 2 and 4 and Supporting information, Figure S3). The four cases of $p16^{+}/HPV^{-}$ adenocarcinomas included three of 14 (21%) cases of gastric type and one of 10 (10%) case of clear cell type adenocarcinomas. All 25 HPV-independent adenocarcinomas showed preserved Rb expression irrespective of the p16 status.

For the control group (endometrioid carcinomas and serous carcinomas of endometrial or equivocal origin), all 13 cases were negative for HR-HPV and six of 13 (46%) were positive for p16; however, all showed preserved Rb expression pattern (Figures 4 and 5). In this group, all six cases with p16 positivity were serous carcinoma (see below result).

### RELIABILITY OF P16 AND RB EXPRESSION PATTERNS TO PREDICT HPV INFECTION

The 108 primary ECAs were divided into $p16^{+}/HPV^{+}$ ($n$ = 83, 77%), $p16^{+}/HPV^{-}$ ($n$ = four, 4%) and $p16^{-}/HPV^{-}$ ($n$ = 21, 19%) groups (Figure 4). Among the p16-positive cases ($n$ = 87), tumours with partial-loss pattern of Rb were exclusively HPV-positive ($n$ = 83) and those with Rb-preserved pattern were exclusively HPV-negative ($n$ = four). All 21 cases with p16 negativity showed preserved Rb pattern and lacked HPV infection. Thirteen cases of the control group were divided into $p16^{+}/HPV^{-}$ ($n$ = six, 46%) and $p16^{-}/HPV^{-}$ ($n$ = seven, 54%) groups (Figure 4). All 13 cases showed preserved Rb patterns.

Compared with p16, the combination of p16 overexpression and Rb-partial loss showed equally excellent sensitivity (each 100%) and improved specificity (100 versus 84%) and positive predictive values (100 versus 95.4%) in ECAs (Table 2). Similarly, the combined use of p16 and Rb patterns revealed excellent sensitivity (each 100%) and improved specificity (100 versus 73.6%) and positive predictive values (100 versus 89.2%) in the ECA and control groups (Table 2).

### HISTOPATHOLOGICAL FINDINGS OF HPV-ASSOCIATED AIS AND ADENOCARCINOMA

Representative histopathological images of HPV-associated AIS, conventional type ($n$ = three), and SMILE ($n$ = two) are shown in Supporting information, Figure S1.

Among 78 HPV-associated adenocarcinomas, the conventional usual type ($n$ = 60), micropapillary pattern ($n$ = one), villoglandular variants ($n$ = six), mucinous type NOS ($n$ = five), intestinal adenocarcinomas ($n$ = two), signet-ring cell adenocarcinomas ($n$ = two) and stratified mucin-producing carcinomas ($n$ = two) were predominant histopathological patterns (Figure 1, Supporting information, Table S2). These cases showed consistent histological appearances and immunohistochemical profiles. An exceptional case of usual type adenocarcinoma was positive for ER and PgR simulating endometrioid adenocarcinoma; otherwise, features such as 'floating mitosis', p16 positivity and Rb partial loss pattern were the same as conventional usual type HPV-associated adenocarcinomas (Supporting information, Figure S2). Adenocarcinomas with predominant micropapillary pattern superficially resembled serous carcinomas in terms of confluent growth with micropapillary tufting; however, the presence of HPV infection avoided the diagnosis of serous carcinomas (Figure 1).

### HISTOPATHOLOGICAL FINDINGS OF HPV-INDEPENDENT ADENOCARCINOMAS

Gastric type HPV-independent adenocarcinomas ($n$ = 14) showed consistent histopathological features

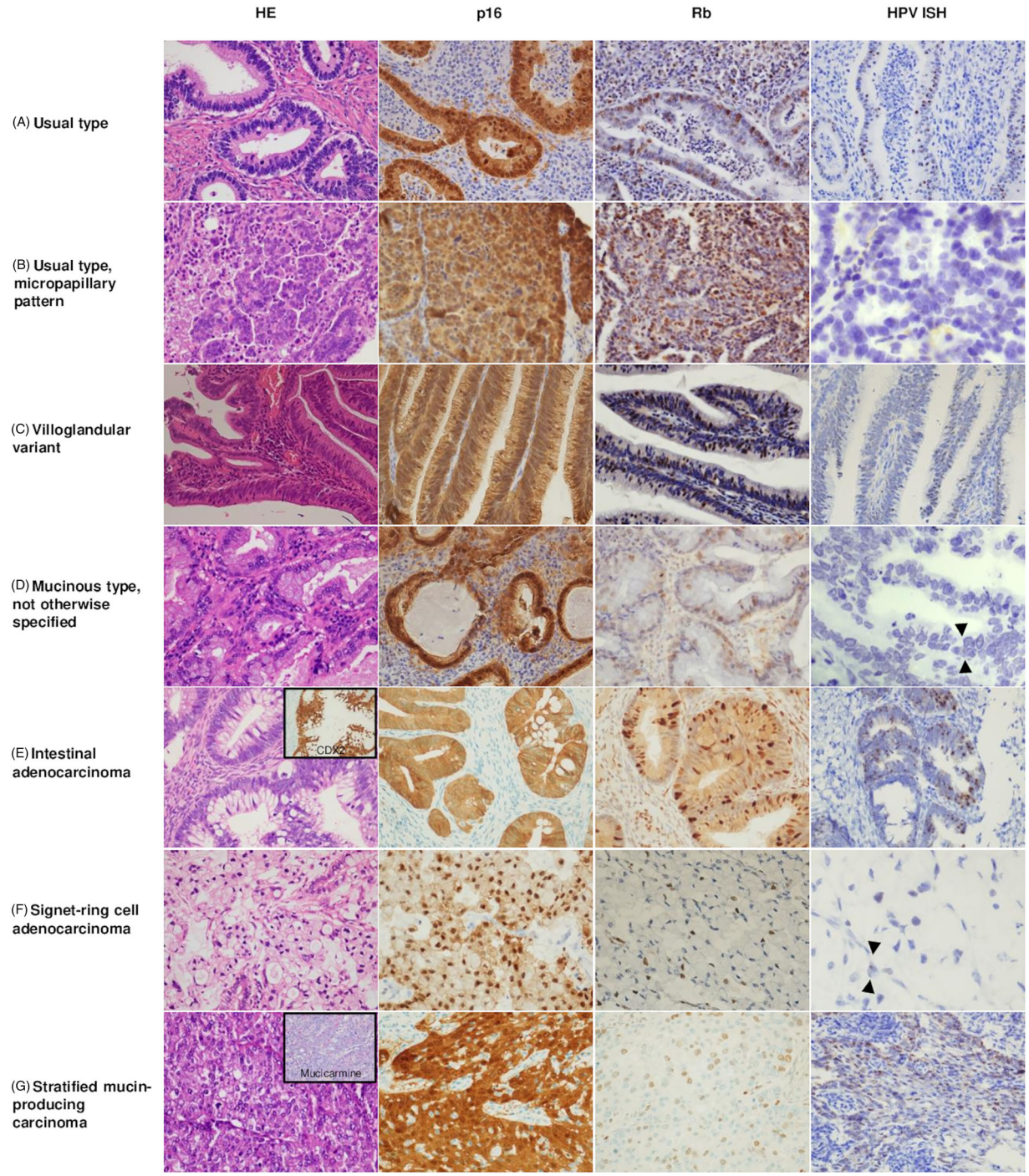


**Figure 1.** Representative histology, immunohistochemistry for p16 and Rb and *in-situ* hybridisation (ISH) for high-risk human papillomavirus (HR-HPV) in invasive HPV-associated adenocarcinoma of the uterine cervix. **A–G,** All subtypes/variants show a diffuse nuclear and cytoplasmic expression ('block' positivity) of p16, partial loss of Rb and various degrees of positive signals for HR-HPV mRNA ISH. **A,** Usual type. Well-formed glandular proliferation of adenocarcinoma cells with conspicuous apical mitoses. **B,** Usual type, micropapillary pattern. Confluent growth of carcinoma cells with micropapillary tufting. **C,** Villoglandular variant. Exophytic papillary growth of mucin-depleted, tall columnar cells. **D,** Mucinous type, not otherwise specified, adenocarcinoma. Cribriform proliferation of the atypical mucinous epithelium with pale cytoplasm. **E,** Intestinal adenocarcinoma. Glandular proliferation of goblet cell-type atypical epithelium. Tumour cells show diffuse CDX2 expression (inset). **F,** Signet-ring cell adenocarcinoma. Discohesive proliferation of carcinoma cells with intracytoplasmic mucinous vacuole and peripherally located nuclei. **G,** Stratified mucin-producing carcinoma. Sheet-like proliferation of carcinoma cells with eosinophilic to pale cytoplasm. Mucicarmine staining revealed intracytoplasmic mucin (inset).

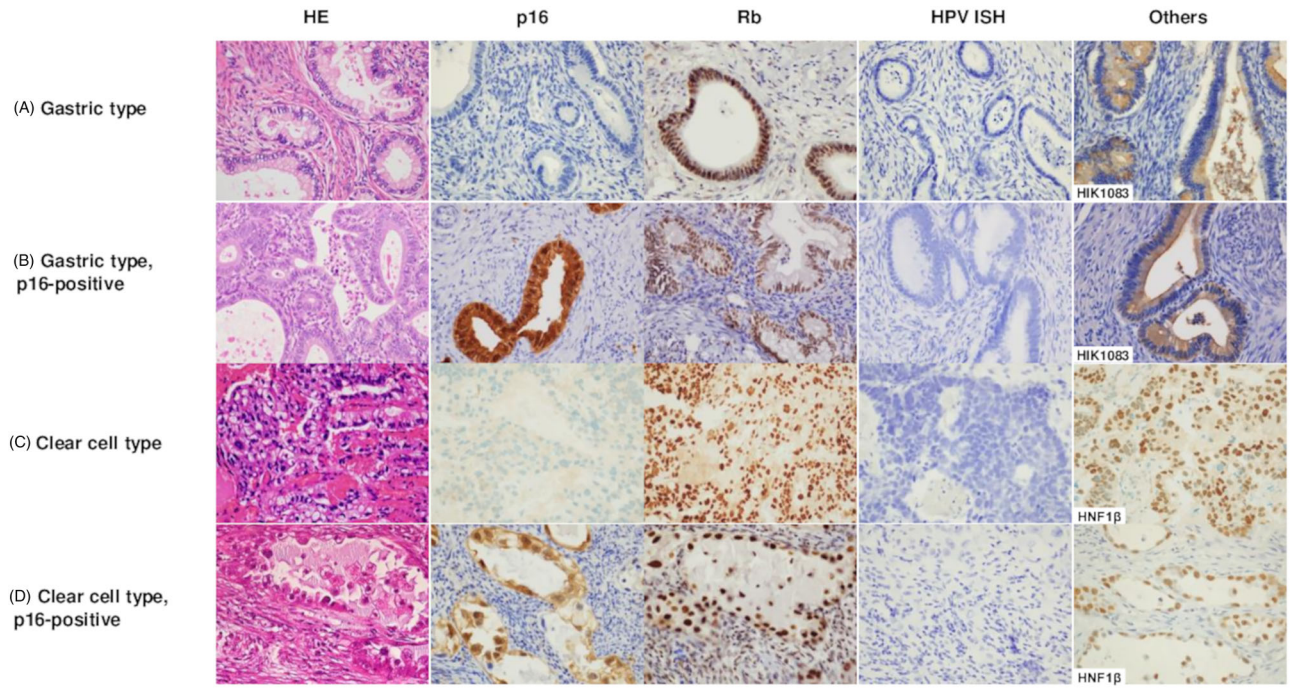


**Figure 2.** Representative histology, immunohistochemistry for p16 and Rb and *in-situ* hybridisation (ISH) for high-risk human papillomavirus (HR-HPV) in invasive HPV-independent adenocarcinoma of the uterine cervix. **A–D,** All subtypes show preserved Rb expression and are negative for HR-HPV mRNA ISH. **A,** Gastric type. Adenocarcinoma cells with pale cytoplasm proliferate in well-differentiated glandular structure. Typically, p16 is negative and HIK1083 is positive. **B,** Gastric type, p16-positive case. p16 is positive in a subset of gastric type adenocarcinomas; otherwise, features are the same as p16-negative cases. **C,** Clear cell type. Tumour cells are arranged in papillary, glandular and solid patterns, lined by cells with clear and eosinophilic cytoplasms. Typically, p16 is negative and HNF1β is positive. **D,** Clear cell type, p16-positive case. p16 is positive in a subset of clear cell type adenocarcinomas; otherwise, features are the same as p16-negative cases.

characterised by glandular proliferation of atypical epithelial cells with abundant clear or pale eosinophilic cytoplasm and distinct cell borders,[2] as well as immunopositivity for HIK1083 (Figure 2). Three of 14 (21%) cases were positive for p16. Rb expression was preserved in all 14 cases irrespective of the p16 status (Table 1). Clear cell type HPV-independent adenocarcinomas ($n$ = 10) showed consistent histopathological features; i.e. the carcinoma cells with clear, eosinophilic or granular cytoplasm proliferate in tubulocystic, papillary and/ or solid architecture sometimes with hobnail pattern,[2] as well as immunopositivity for HNF1β (Figure 2). One of 10 (10%) cases was positive for p16, but still showed preserved Rb expression (Table 1). Only one patient had HPV-independent adenocarcinoma, NOS with peculiar morphology; i.e. the tumour cells with eosinophilic or pale cytoplasm were arranged in irregular glandular, cribriform pattern and solid pattern (Figure 3). Intracytoplasmic mucin was observed. The tumour existed in the uterine cervix and did not affect the endometrium. This case was negative for the markers often seen in other type ECAs, including ER, PgR, CDX2, HIK1083, HNF1β and GATA3, and showed scattered immunoreactivity for p53 consistent with wild-type expression pattern (Supporting information, Figure S3).

### HISTOPATHOLOGICAL FINDINGS OF ENDOMETRIOID CARCINOMAS AND SEROUS CARCINOMAS OF ENDOMETRIAL OR EQUIVOCAL ORIGIN

All six cases of endometrioid carcinomas were positive for ER and PgR but negative for p16 and HR-HPV (Figure 5). Rb expression was preserved. The majority (six of seven cases; 86%) of serous carcinomas were positive for p16; however, HR-HPV was negative, and Rb expression was preserved (Table 1). Aberrant expression of p53 was seen in all seven cases, including diffuse ($n$ = four) or null ($n$ = three) pattern.

### CLINICOPATHOLOGICAL AND PROGNOSTIC ASSOCIATION OF HPV INFECTION

HR-HPV infection was significantly associated with younger age, lower stage, lower rates of lymph node

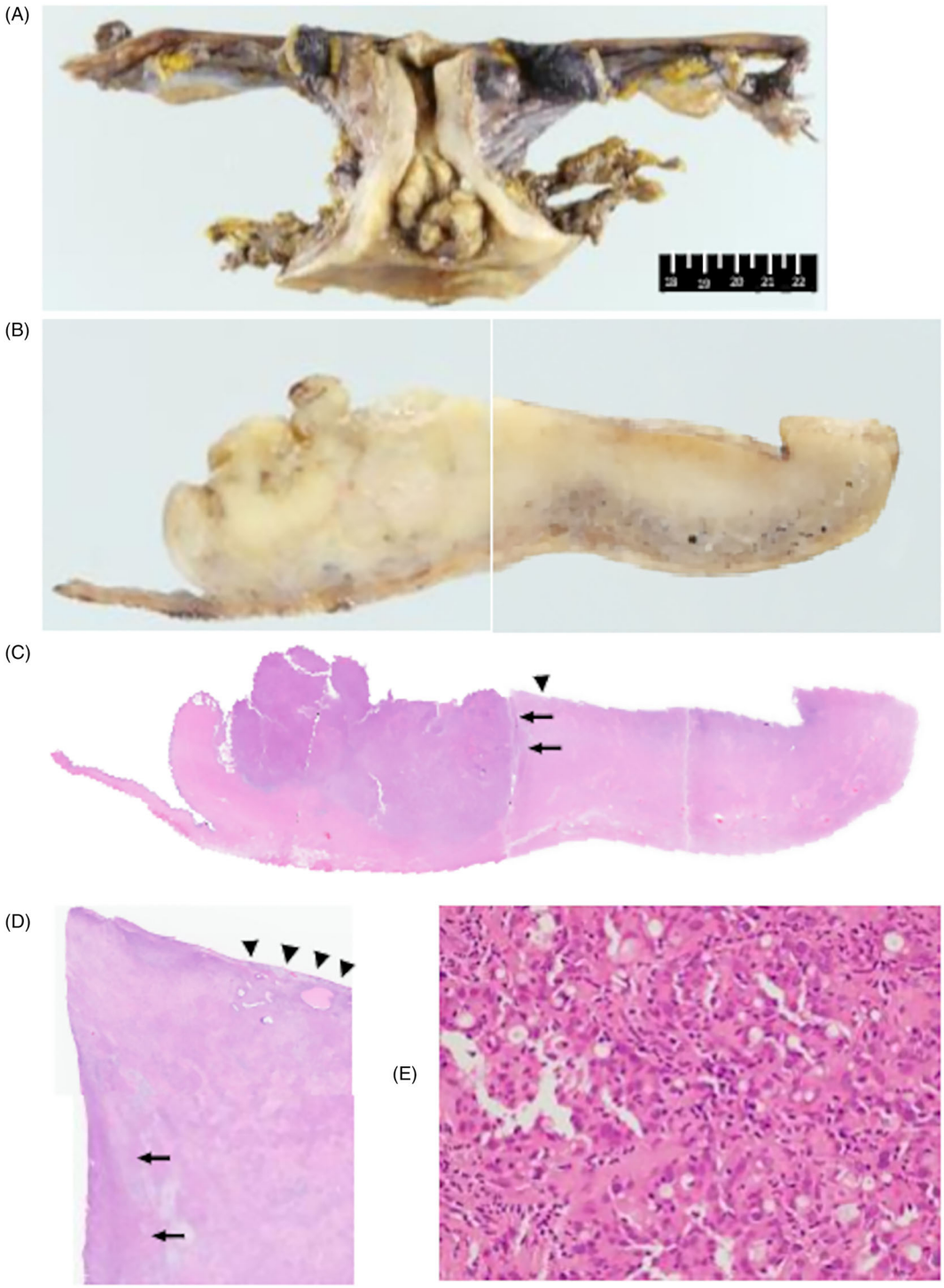


**Figure 3.** A case of invasive, HPV-independent, not otherwise specified, adenocarcinoma of the uterine cervix. **A,** On gross appearance, the protruded tumour is located in the uterine cervix. **B,** The cross-section demonstrates a tumour mainly involving the endocervix. **C,D,** The corresponding panoramic view of histology shows that the tumour (arrows) is apart from the endometrium (arrowheads). **E,** Tumour cells are arranged in irregular glandular and cribriform patterns, lined by cells with eosinophilic cytoplasm. Cytoplasmic mucin is observed.

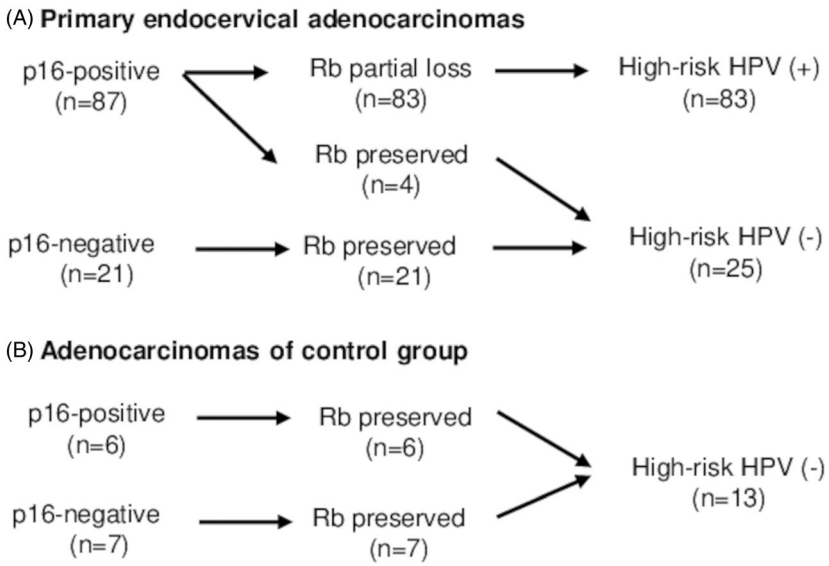


**Figure 4.** Mutual relationship among p16 expression, Rb status and high-risk human papillomavirus (HR-HPV) infection in uterine cervical adenocarcinomas. **A,** Primary endocervical adenocarcinomas. Among p16-positive cases, the partial loss pattern of Rb was absolutely associated with HR-HPV infection, whereas cases with preserved pattern of Rb were negative for HR-HPV. All p16-negative cases showed a preserved pattern of Rb and absence of HR-HPV infection. **B,** Adenocarcinomas of the control group (endometrioid carcinoma and serous carcinoma, and invasion from the endometrium or undetermined primary site). All cases, irrespective of p16 status, showed preserved expression of Rb and absence of HR-HPV infection.

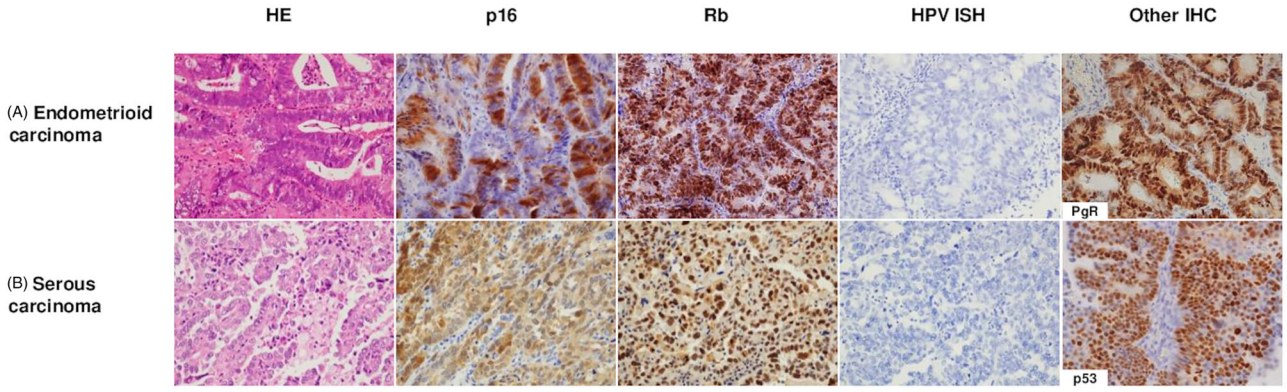


**Figure 5.** Representative histology, immunohistochemistry for p16 and Rb and *in-situ* hybridisation (ISH) for high-risk human papillomavirus (HR-HPV) in invasive adenocarcinomas of the control group (endometrioid carcinoma and serous carcinoma, invasion from endometrial cancer or undetermined primary site). **A,** Endometrioid carcinomas. Mucin-depleted, atypical columnar cells with hyperchromatic nuclei proliferate in well-formed or fused glandular patterns, superficially mimicking the usual type of adenocarcinomas. Apical mitoses and apoptotic bodies were not observed. p16 expression can be focal but not 'block' positive, judged as negative result. Rb expression is preserved and HR-HPV is negative. Tumour cells are diffuse positive for PgR. **B,** Serous carcinoma of the endometrium. Tumour cells are arranged in micropapillary patterns, lined by cells with high-grade nuclear atypia, superficially mimicking the micropapillary pattern of the usual type of adenocarcinoma. p16 is diffusely expressed, judged as positive. Rb expression is preserved, and HR-HPV is negative. Tumour cells show aberrant, p53 overexpression.

metastasis and lower rates of recurrence ($P < 0.0001$, $P = 0.0396$, $P = 0.0115$, and $P = 0.0450$, respectively; Supporting information, Table S3).

In the Kaplan–Meier estimate using log-rank tests, HPV infection was significantly associated with longer OS among patients with invasive ECAs ($P = 0.0254$; Figure 6). When cases were limited to advanced stage ECAs, HPV-positive cases tended to have longer OS ($P = 0.0534$; Figure 6). HPV-associated adenocarcinomas of usual type had significantly better prognosis

**Table 2.** The reliability of immunohistochemical expression pattern of p16 and Rb to predict HR-HPV infection

| | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| ECAs ($n$ = 108) | | | | |
| p16$^{+}$ alone | 100 | 84 | 95.4 | 100 |
| p16+/Rb partial loss | 100 | 100 | 100 | 100 |
| ECAs and adenocarcinoma of endometrium or equivocal origin ($n$ = 121) | | | | |
| p16$^{+}$ alone | 100 | 73.6 | 89.2 | 100 |
| p16+/Rb partial loss | 100 | 100 | 100 | 100 |

ECA, endocervical adenocarcinoma; HR, high-risk; HPV, human papillomavirus; NPV, negative predictive value; PPV, positive predictive value.

than HPV-independent adenocarcinomas of gastric and clear cell types ($P$ = 0.0070; Supporting information, Figure S4).

In the univariate analysis using the Cox proportional hazards model, age (≥ 50 years, $P$ = 0.0150), stage (III + IV, $P$ = 0.0003), lymph node metastases (positive, $P$ < 0.0001), HR-HPV status (negative, $P$ = 0.0349) and preserved Rb pattern ($P$ = 0.0349) significantly correlated with shorter OS among patients with invasive ECAs (Supporting information, Table S4).

## Discussion

The incidence of HPV infection in ECAs is gaining more attention because the fifth WHO classification clearly divides ECAs into HPV-associated and HPV-independent tumours. In this study, HR-HPV infection was detected in 76% of the invasive ECAs, in agreement with previous studies worldwide (57–90%).[3,4,10]

Most ECAs are aetiologically associated with HPV clades A9 (typically HPV16) and A7 (typically HPV18). HPV viral oncoproteins E6 and E7 inactivate p53 and Rb, respectively. This inactivation is associated with the integration of the HPV genome into the host genome, leading to the genomic instability and accumulation of somatic mutations.[27] The E7-binded Rb protein is degraded through the ubiquitin–proteasome pathway.[15]

Optimal HPV testing in the practical diagnosis of ECAs is not yet established because of the advantages and disadvantages in any testing methods. HR-HPV mRNA chromogenic ISH, recognising 18 HR-HPV types, is approximately as sensitive as p16 IHC and is more specific;[4] however, this test is not yet widely available for clinical use in several countries, including Japan. Although p16 IHC is a highly sensitive and inexpensive test for HR-HPV infection, standalone p16 is not a perfect surrogate marker.[4] In this series, four of 25 (16%) HPV-independent adenocarcinomas showed a diffuse, 'block' positivity for p16 despite the consistent morphology of gastric or clear cell type adenocarcinomas. In this study, the combination of p16 overexpression and Rb partial loss pattern were closely associated with HR-HPV infection in ECAs and showed excellent sensitivity, specificity and positive predictive values.

The result indicates that the p16/Rb IHC panel may be a reliable method to predict HR-HPV infection in ECAs, especially in situations in which HPV-ISH is not available. A similar trend has been demonstrated in SCCs in the sinonasal tract,[16] oropharynx,[17] conjunctiva and lacrimal sac.[18] In our previous study of oropharyngeal SCC we conducted blinded scoring of Rb to check the interobserver variability, and revealed high reproducibility of Rb interpretation.[17] Moreover, in our preliminary study, HR-HPV infection was closely associated with the partial loss (mosaic) pattern of Rb IHC in HPV-associated SCCs of the uterine cervix (unpublished data), suggesting that the combination of p16 and Rb expression patterns might represent a useful surrogate for HR-HPV infection among the histological types; however, further study is needed to reach a definitive conclusion.

For the differential diagnosis, the combined use of p16 and Rb may help in histological typing of ECAs, particularly in cases with equivocal morphology and/or unusual result of phenotypical markers. Examples of diagnostic dilemmas are as follows:

1. HPV-independent adenocarcinomas of gastric type can be positive for p16 (as mentioned above). In addition, this type of cancer can possess focal overlapping morphology with HPV-associated adenocarcinomas of usual type.[2,6,28]
2. HPV-associated adenocarcinomas of usual type can resemble endometrioid adenocarcinomas in terms of histomorphology and ER/PgR expression.[29] Reportedly, ER and PgR were expressed in 5 and 20% of HPV-associated adenocarcinomas, usual type.[12] It is well known that endometrioid adenocarcinoma of the endometrium is usually positive for ER and PgR,[30] but the data on endometrioid adenocarcinoma of true endocervical origin are extremely limited because of its rarity. According to a large series of ECAs which were strictly classified according to the HPV status,

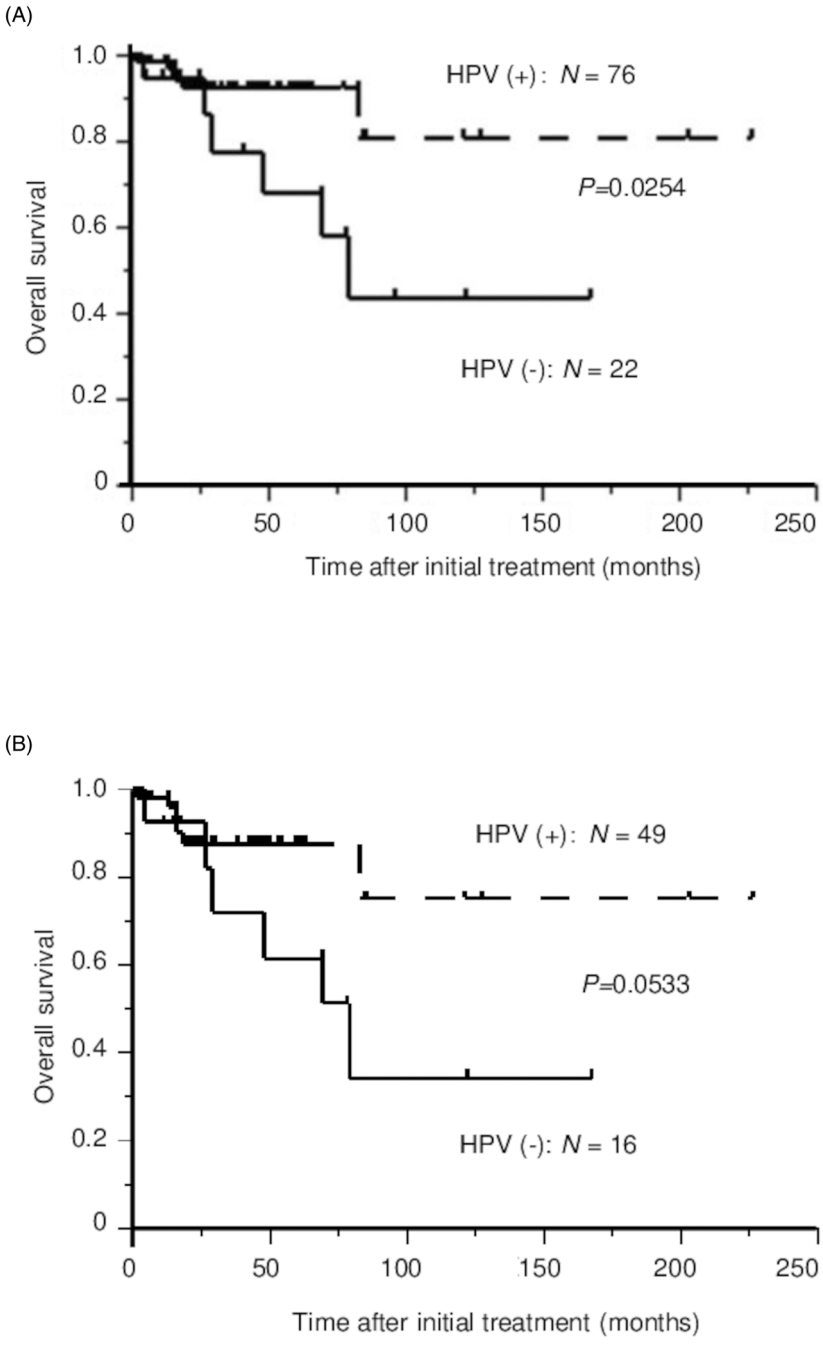


**Figure 6.** Prognostic analyses in invasive endocervical adenocarcinomas (ECAs) according to high-risk human papillomavirus (HR-HPV) infection. **A,** HR-HPV infection in invasive ECAs is significantly associated with longer overall survival ($P$ = 0.0254). **B,** HR-HPV infection tends to have longer overall survival period in invasive ECAs of advanced clinical stage (> stage IB1; $P$ = 0.0533).

ER and PgR expressions were present in each 33% (one of three cases) of HPV-independent endometrioid adenocarcinomas of the uterine cervix.[12]

3. HPV-associated adenocarcinomas with micropapillary pattern can share the features of p16 positivity and morphology with serous carcinomas.[2,4] It is reported that aberrant expression of p53 was seen in most cases of serous carcinoma,[14] but only 3.6% of HPV-associated adenocarcinomas, usual type.[12] When p16 staining is diffusely positive, p53 IHC could be helpful in the differential diagnosis in addition to Rb IHC.

4. HPV-associated adenocarcinoma, mucinous type can exhibit overlapping features with HPV-independent adenocarcinoma, gastric type concerning the presence of intracytoplasmic mucin and p16 positivity.[2,3,6] In addition, caution is needed because p53 aberrant expression has been reported in 18% of HPV-associated adenocarcinomas, mucinous type and 52% of HPV-independent adenocarcinomas, gastric type.[12]

In this study, endometrioid adenocarcinomas and serous carcinomas of true endocervical origin did not exist, and the result was consistent with previous reports.[2] Most cases of serous carcinomas of endometrial or equivocal origin were positive for p16; however, they were prone to have the features of HPV-negative and preserved Rb pattern. Thus, in addition to the aid of p16/Rb IHC, the recognition of the prevalence of each ECA subtype may help for differential diagnosis.

Only one case of HPV-independent adenocarcinoma, NOS, was noted in our series. This case showed peculiar histological appearance and uncharacteristic immunohistochemical profile (Figure 3 and Supporting information, Figure S3). Further studies are needed to characterise the clinicopathological features of rare subtypes of HPV-independent ECAs.

Regarding biological behaviour, HPV-independent tumours are generally more aggressive than their HPV-associated counterparts among oropharyngeal and sinonasal SCCs.[16,17,31,32] The prognostic significance of HR-HPV infection remains controversial in ECAs. According to Stolnicu *et al*., a nearly significant association between HPV infection and favourable prognosis exists among patients with ECAs on multivariate analysis.[33] Hodgson *et al*. also reported that HPV infection in ECAs correlated with worse disease-free and disease-specific survival on the univariate analysis.[8] Kojima *et al*. reported that only gastric type HPV-independent adenocarcinomas had a significantly higher risk for disease recurrence than other types of ECAs.[6] In the present study, HPV infection correlated with better prognosis in the univariate analysis. In addition, HPV-associated adenocarcinomas of usual type had significantly better prognosis than HPV-independent adenocarcinomas, including gastric and clear cell types ($P = 0.0070$). The results support the rationale of the current fifth WHO classification emphasising HPV infection.

In conclusion, the findings of this study suggest that p16-IHC and Rb-IHC combination may be a useful surrogate for the detection of transcriptionally active HR-HPV in ECAs. Compared with p16 alone, this combination IHC may become a helpful adjunct for the more accurate stratification of patients with ECAs. In this context, we propose inclusion of Rb-IHC into the diagnostic algorithm for ECAs and adenocarcinomas of equivocal origin in the routine clinical practice. In addition, the subclassification of ECAs based on HPV infection might provide important information for prognostic prediction and therapeutic strategies.

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## Conflicts of interest

The authors have no potential conflicts of interest to disclose.

## Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

## References

1. Walboomers JM, Jacobs MV, Manos MM *et al*. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* 1999; **189**; 12–19.
2. Herrington CSKK-R, Kong CS, Longacre TA *et al*. *Tumours of the uterine cervix. WHO classification of Tumours editorial board. Female genital tumors*. 5th ed. Lyon: International Adency for Research on Cancer, 2020; 336–389.
3. Park KJ, Kiyokawa T, Soslow RA *et al*. Unusual endocervical adenocarcinomas: an immunohistochemical analysis with molecular detection of human papillomavirus. *Am. J. Surg. Pathol.* 2011; **35**; 633–646.
4. Stolnicu S, Barsan I, Hoang L *et al*. International Endocervical Adenocarcinoma Criteria and Classification (IECC): a new pathogenetic classification for invasive adenocarcinomas of the endocervix. *Am. J. Surg. Pathol.* 2018; **42**; 214–226.
5. Wilbur DC, Colgan TJ, Ferenczy AS *et al*. Glandular tumors and precursors. In Kurman RJ, Carcangiu ML, Herrington CS, Young RH eds. *WHO classification of Tumours of female*

*reproductive organs*. 4th ed. Lyon: International Agency for Research on Cancer, 2014; 183–194.
6. Kojima A, Mikami Y, Sudo T *et al*. Gastric morphology and immunophenotype predict poor outcome in mucinous adenocarcinoma of the uterine cervix. *Am. J. Surg. Pathol.* 2007; **31**; 664–672.
7. Kusanagi Y, Kojima A, Mikami Y *et al*. Absence of high-risk human papillomavirus (HPV) detection in endocervical adenocarcinoma with gastric morphology and phenotype. *Am. J. Pathol.* 2010; **177**; 2169–2175.
8. Hodgson A, Olkhov-Mitsel E, Howitt BE, Nucci MR, Parra-Herran C. International Endocervical Adenocarcinoma Criteria and Classification (IECC): correlation with adverse clinicopathological features and patient outcome. *J. Clin. Pathol.* 2019; **72**; 347–353.
9. Huss A, Ihorst G, Timme-Bronsert S, Hasenburg A, Oehler MK, Klar M. The Memorial Sloan Kettering Cancer Center nomogram is more accurate than the 2009 FIGO staging system in the prediction of overall survival in a German endometrial cancer patient cohort. *Ann. Surg. Oncol.* 2018; **25**; 3966–3973.
10. Houghton O, Jamison J, Wilson R, Carson J, McCluggage WG. p16 immunoreactivity in unusual types of cervical adenocarcinoma does not reflect human papillomavirus infection. *Histopathology* 2010; **57**; 342–350.
11. Ueno S, Sudo T, Oka N *et al*. Absence of human papillomavirus infection and activation of PI3K-AKT pathway in cervical clear cell carcinoma. *Int. J. Gynecol. Cancer* 2013; **23**; 1084–1091.
12. Stolnicu S, Barsan I, Hoang L *et al*. Diagnostic algorithmic proposal based on comprehensive immunohistochemical evaluation of 297 invasive endocervical adenocarcinomas. *Am. J. Surg. Pathol.* 2018; **42**; 989–1000.
13. Milea A, George SH, Matevski D *et al*. Retinoblastoma pathway deregulatory mechanisms determine clinical outcome in high-grade serous ovarian carcinoma. *Mod. Pathol.* 2014; **27**; 991–1001.
14. Yemelyanova A, Ji H, Shih Ie M *et al*. Utility of p16 expression for distinction of uterine serous carcinomas from endometrial endometrioid and endocervical adenocarcinomas: immunohistochemical analysis of 201 cases. *Am. J. Surg. Pathol.* 2009; **33**; 1504–1514.
15. Boyer SN, Wazer DE, Band V. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res.* 1996; **56**; 4620–4624.
16. Jiromaru R, Yamamoto H, Yasumatsu R *et al*. HPV-related sinonasal carcinoma: clinicopathologic features, diagnostic utility of p16 and Rb immunohistochemistry, and EGFR copy number alteration. *Am. J. Surg. Pathol.* 2020; **44**; 305–315.
17. Jiromaru R, Yamamoto H, Yasumatsu R *et al*. p16 overexpression and Rb loss correlate with high-risk HPV infection in oropharyngeal squamous cell carcinoma. *Histopathology* 2021; **79**; 358–369.
18. Hongo T, Yamamoto H, Tanabe M *et al*. High-risk HPV-related squamous cell carcinoma in the conjunctiva and lacrimal sac: clinicopathologic characteristics and diagnostic utility of p16 and Rb immunohistochemistry. *Am. J. Surg. Pathol.* 2022; **46**; 977–987.
19. Rickman DS, Beltran H, Demichelis F, Rubin MA. Biology and evolution of poorly differentiated neuroendocrine tumors. *Nat. Med.* 2017; **23**; 1–10.
20. Umetsu SE, Kakar S, Basturk O *et al*. Integrated genomic and clinicopathologic approach distinguishes pancreatic grade 3 neuroendocrine tumor from neuroendocrine carcinoma and identifies a subset with molecular overlap. *Mod. Pathol.* 2023; **36**; 100065.
21. Febres-Aldana CA, Chang JC, Ptashkin R *et al*. Rb tumor suppressor in small cell lung cancer: combined genomic and IHC analysis with a description of a distinct Rb-proficient subset. *Clin. Cancer Res.* 2022; **28**; 4702–4713.
22. Weigelt B, Marra A, Selenica P *et al*. Molecular characterization of endometrial carcinomas in black and white patients reveals disparate drivers with therapeutic implications. *Cancer Discov.* 2023; **13**; 2356–2369.
23. The Cancer Genome Atlas Program. Updated 19 January 2024. https://portal.gdc.cancer.gov.
24. Xiao Z, He Y, Liu C *et al*. Targeting P16INK4A in uterine serous carcinoma through inhibition of histone demethylation. *Oncol. Rep.* 2019; **41**; 2667–2678.
25. Vermorken JB, Psyrri A, Mesía R *et al*. Impact of tumor HPV status on outcome in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck receiving chemotherapy with or without cetuximab: retrospective analysis of the phase III EXTREME trial. *Ann. Oncol.* 2014; **25**; 801–807.
26. Bishop JA, Ma XJ, Wang H *et al*. Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA in situ hybridization method. *Am. J. Surg. Pathol.* 2012; **36**; 1874–1882.
27. Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. *Nat. Rev. Cancer* 2010; **10**; 550–560.
28. Wada T, Ohishi Y, Kaku T *et al*. Endocervical adenocarcinoma with morphologic features of both usual and gastric types: clinicopathologic and immunohistochemical analyses and high-risk HPV detection by in situ hybridization. *Am. J. Surg. Pathol.* 2017; **41**; 696–705.
29. Chen R, Qin P, Luo Q *et al*. ER-positive endocervical adenocarcinoma mimicking endometrioid adenocarcinoma in morphology and immunohistochemical profile: a case report of application of HPV RNAscope detection. *Medicine (Baltimore)* 2021; **100**; e24927.
30. Lim D, Ip PP, Cheung AN, Kiyokawa T, Oliva E. Immunohistochemical comparison of ovarian and uterine endometrioid carcinoma, endometrioid carcinoma with clear cell change, and clear cell carcinoma. *Am. J. Surg. Pathol.* 2015; **39**; 1061–1069.
31. Nakano T, Yamamoto H, Nakashima T *et al*. Molecular subclassification determined by human papillomavirus and epidermal growth factor receptor status is associated with the prognosis of oropharyngeal squamous cell carcinoma. *Hum. Pathol.* 2016; **50**; 51–61.
32. Hongo T, Yamamoto H, Jiromaru R *et al*. Clinicopathologic significance of EGFR mutation and HPV infection in sinonasal squamous cell carcinoma. *Am. J. Surg. Pathol.* 2021; **45**; 108–118.
33. Stolnicu S, Hoang L, Chiu D *et al*. Clinical outcomes of HPV-associated and unassociated endocervical adenocarcinomas categorized by the International Endocervical Adenocarcinoma criteria and Classification (IECC). *Am. J. Surg. Pathol.* 2019; **43**; 466–474.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Representative histology, immunohistochemistry for p16 and Rb, and *in-situ* hybridisation

(ISH) for high-risk human papillomavirus (HR-HPV) in HPV-associated, adenocarcinoma in situ (AIS) of the uterine cervix. (A, B) All subtypes show diffuse nuclear and cytoplasmic expression ('block' positivity) of p16, partial loss of Rb, and various degrees of positive signals for HR-HPV mRNA ISH. **A**, Conventional AIS. Atypical columnar cells with depleted mucin and nuclear hyperchromasia proliferate in a glandular pattern. **B**, Stratified mucin-producing intraepithelial lesion. Stratified, mucinous neoplastic cells proliferate within the epithelium.

**Figure S2.** A case of uterine cervical, HPV-associated adenocarcinoma with aberrant ER and PgR expressions. **A**, Invasive adenocarcinoma cells with mucin-depleted cytoplasm and hyperchromatic nuclei proliferate in irregular glandular and cribriform patterns, superficially mimicking endometrioid adenocarcinomas. Conspicuous apical mitoses and apoptosis are present. **B**, Diffuse p16 expression. **C**, Partial loss of Rb. **D**, HR-HPV infection by *in-situ* hybridisation. **E**, ER positive. **F**, PgR positive.

**Figure S3.** A case of invasive, HPV-independent, not otherwise specified, adenocarcinoma of the uterine cervix. **A**, Patchy p16 expression. **B**, Preserved Rb expression. **C**, Absence of HR-HPV infection. **D**, Scattered, wild-type expression of p53. **E–H**, Negative staining for ER (E), PgR (F), HIK1083 (G), and GATA3 (H).

**Figure S4.** Prognostic analysis of uterine cervical adenocarcinomas, HPV-associated, usual type versus HPV-independent, gastric/clear cell types. The former is significantly associated with longer overall survival than the latter ($P = 0.0070$).

**Table S1.** Clinical features of patients with uterine cervical adenocarcinoma.

**Table S2.** Predominant histological patterns of 78 cases of HPV-associated adenocarcinoma.

**Table S3.** Association between clinicopathological factors and HR-HPV infection in 108 cases of uterine cervical adenocarcinomas.

**Table S4.** Univariate and multivariate analyses of overall survival in 103 cases of invasive uterine cervical adenocarcinoma.