

Original Article

Detection of high-risk human papillomavirus subtypes in cervical glandular neoplasia by in situ hybridization

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Abstract: In situ hybridization (ISH) was performed on paraffin-embedded tissues to detect multiple high-risk human papillomavirus (HPV) subtypes in 27 cases of cervical adenocarcinoma in situ (AIS) and adenocarcinoma (CA) specimens. These results were compared with those of HPV detection by HPV-PCR genotyping and p16 immunohistochemistry in the same specimens. Of the 27 cases, 17 (63%) showed HPV-DNA by HPV-ISH, including 3 metastatic lesions. HPV-DNA was detected in 18 cases (67%) by PCR. The concordance rate between HPV-ISH and HPV-PCR genotyping was 74% with moderate agreement (Kappa value, 0.41). HPV-16 was identified in 5 cases, HPV-18 in 2 cases, and HPV-45 in 1 case. Combining the results of HPV-ISH and HPV-PCR/genotyping, 22 cases (81.5%) were considered HPV positive. Immunohistochemical staining of p16 indicated that 25 (93%) cases were positive; however, 4 of these cases were HPV-negative by both PCR and ISH. Combining HPV-ISH and HPV-PCR/genotyping techniques demonstrated a high sensitivity of HPV detection in FFPE tissues from cervical glandular neoplasias. In contrast, p16 immunohistochemistry seemed to have a low specificity for determining HPV status in cervical glandular neoplasia. HPV-ISH is useful for recognizing the distribution of HPV in AIS and CA tissues and visualizing signal patterns, and may be a useful tool to confirm the cervical origin of neoplasias and metastatic lesions.

Keywords: Uterine cervical cancer, adenocarcinoma, human papillomavirus, in situ hybridization, PCR, p16^{INK4A}

Introduction

The pathogenesis of cervical adenocarcinoma (CA) is not fully understood. CA is a heterogeneous group of tumors. Although human papillomavirus (HPV) is undoubtedly the primary cause of cervical cancer (regardless of the histological subtype involved) [1-3], unusual variants may not be related to HPV infection [4, 5].

HPV detection in cervical lesions has typically been achieved using currently available Hybrid Capture 2 assay or polymerase chain reaction (PCR)-based HPV type-specific DNA tests. In situ hybridization (ISH) for HPV DNA (HPV-ISH) has recently become more readily available in most laboratories as a routine diagnostic test [6]. In contrast to PCR-based assays, a particular advantage of ISH is that it allows visualization of the virus within particular lesions. However, relatively few studies have included HPV-ISH assays for cervical cancers, especially

in adenocarcinoma cases. Of these, some studies have used probes limited to the high-risk subtypes HPV-16 and/or HPV-18 [7-10] or isotope-labeled probes [9, 11]. Most studies have used HPV-ISH for the discrimination of CA from endometrial cancer [12-14], to study adenocarcinoma in situ [15], or in a case study [11]. The rates of HPV-ISH positive results in these studies varied from 40% to 85% [7], and some even reported negative results [8]. Additionally, immunohistochemistry (IHC) of p16^{INK4A} (p16) has been widely used for the detection of high-risk HPV in cervical squamous lesions, and some studies have demonstrated that this method has a higher sensitivity than HPV-ISH in squamous lesions [16, 17]. However, Milde-Langosch et al. reported p16 expression in 41% of HPV-negative CAs [9], and Houghton et al. reported that p16 immunoreactivity in unusual types of CA does not reflect HPV infection [18]. To our knowledge, only a few studies have simultaneously analyzed HPV-PCR genotyping,

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HPV status determined by ISH, and p16 immunoreactivity in CA tissues [9, 12, 19].

In the present study, formalin-fixed paraffin-embedded (FFPE) cervical glandular neoplasms from Japanese patients were used to perform HPV-ISH with probes targeting several high-risk HPV subtypes along with HPV-PCR, genotyping, and p16 IHC assays. We compared the sensitivity and specificity of each method in association with histological findings and evaluated the merits of each technique alone, or in combination, for the detection of HPV in cervical glandular neoplasia.

Materials and methods

Case selection

A total of 27 CA and adenocarcinoma in situ (AIS) cases treated from 2000 to 2011 were consecutively retrieved from pathology files at Kanazawa Medical University. In all cases, the diagnosis was obtained by clinico-radiological-pathological correlation. The specimens included 8 biopsy, 2 conization, and 17 hysterectomy samples. Adjuvant radiation, chemotherapy, or chemoradiation therapy was administered to 15 patients (56%). Patients' ages ranged from 33 to 85 (mean, 60) years.

HPV-ISH

The INFORM HPV VIII Family 16 probe (Ventana Medical Systems, Tucson, AZ, USA), which detects 13 high-risk HPV subtypes (16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, and 70) was used for the study. The ISH assay was performed following the manufacturer's guidelines using the BenchMark automated slide staining system (Ventana Medical Systems). For positive and negative controls, HPV control slides consisting of FFPE sections containing 3 separate collections of cells on a signal slide (Ventana Medical Systems) [6] were used. The HPV signals in nuclei were divided into 2 patterns: a diffuse (or episomal) pattern of condensed and uniformly packed signals, and a dot-like (or integrated) pattern of punctate and sparsely distributed signals. In 3 cases, lymph node metastatic lesions were obtained which were also investigated using ISH.

PCR and genotyping of HPV

DNA was extracted from 5 µm sections of FFPE tissue by microdissection using the Pinpoint

Slide Isolation System (Zymo Research, Orange, CA, USA) according to the manufacturer's guidelines. The extracted DNA was stored at -30°C until use.

The quality of the PCR DNA assay was confirmed by amplifying the b-globin gene as an internal control. HPV-DNA was amplified using a modified GP5⁺/GP6⁺PCR method as previously reported [20]. A positive HPV result was indicated by an approximately 140 bp band after electrophoresis of 5 µl of PCR product on 2.0% agarose gels and staining with ethidium bromide.

HPV genotyping was performed using a GeneSQUARE HPV test (Kurabo, Osaka, Japan). This assay can detect 23 HPV subtypes (6, 11, 16, 18, 30, 31, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, and 68) [21].

p16 IHC

IHC was performed on 4 µm sections of FFPE tissue using a p16 monoclonal antibody (clone 1H4, IBL Co., Gunma, Japan) at a dilution of 1:20. An automated IHC system (Ventana Medical Systems) was used with amplification and detection kits (Ventana Medical Systems). Heat-induced antigen retrieval was performed in a CC1 solution for 30 min. For a positive control, sections of a cervical intraepithelial neoplasia were used with each batch of immunostaining. A positive result was indicated by at least focal (>5%) cytoplasmic or nuclear staining. In 3 cases, metastatic lesions in the lymph node were also investigated by IHC.

Statistical analysis

Kappa statistics were used to assess the agreement between INFORM HPV VIII use for ISH and PCR HPV DNA testing. The chi-square or Fisher's exact test was used to assess the association between categorical variables. *P* values of <0.05 were considered significant with two-sided tests. All statistical analyses were carried out using StatFlex ver 6.0 (Artech, Osaka, Japan).

Results

Clinicopathological features

Twenty-seven tumors were histologically classified according to the current WHO guidelines [1]

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Table 1. Summary of clinicopathological data from studies of HPV in cervical glandular neoplasias

Case	Histology	Age	HPV-ISH	Signal pattern	HPV-PCR	Typing	p16	Stage
1	Mucinous	76	+	dot & diffuse	+	-	+	Ib1
2	Mucinous	52	+	diffuse	+	16	+	Ib1
3	Mucinous	33	+	dot	+	-	+	Ib1
4	Mucinous	63	+	dot & diffuse	+	-	+	IIIb
5	Mucinous	72	+	dot & diffuse	+	-	+	IV
6	Mucinous	66	+	diffuse	-	45	+	IIIb
7	Mucinous	55	-	-	-	-	+	Ib2
8	Mucinous	82	-	-	-	-	-	Ib1
9	Mucinous	58	+	dot & diffuse	+	16	+	Ib1
10	Mucinous	38	-	-	+	16	+	Ib1
11	Mucinous	52	-	-	+	18	+	IIIb
12	Mucinous	71	-	-	+	-	+	Ib1
13	AIS	44	+	dot & diffuse	+	-	+	0
14	AIS (+CIN3)	38	+	diffuse	-	-	-	0
15	AIS (+CIN3)	78	+	diffuse	+	16	+	0
16	AIS (+CIN3)	52	+	dot	-	18	+	0
17	AIS	68	+	diffuse	+	-	+	0
18	AIS	53	-	-	+	-	+	0
19	AIS (+CIN2)	46	+	dot & diffuse	+	16	+	0
20	Serous	67	+	dot	+	-	+	IVb
21	Serous	64	+	diffuse	+	-	+	IVb
22	Clear cell	67	-	-	-	-	f+	Ib1
23	Clear cell (+CIN3)	71	-	-	-	-	f+	IVb
24	Endometrioid	78	-	-	-	-	+	IVa
25	Endometrioid	85	-	-	+	-	+	IIIb
26	Adsq	46	+	dot	-	-	+	IIIb
27	Poor adeno	56	+	diffuse	+	-	+	IVb

AIS: adenocarcinoma in situ, CIN: (squamous) cervical intraepithelial neoplasia, Adsq: adenosquamous carcinoma, poor adeno: poorly differentiated adenocarcinoma, not otherwise specified, f: focal.

as follows: 12 mucinous endocervical adenocarcinomas stages Ib1 (n=4), Ib2 (n=1), IIb (n=3), IIIb (n=3), IVb (n=1); 7 AIS; 2 serous adenocarcinomas stages IIIb and IVa; 2 clear cell adenocarcinomas stages Ib1 and IVb; 2 endometrioid adenocarcinomas stages IIIb and IVa; 1 adenosquamous carcinoma stage IIIb; and 1 poorly differentiated stage IVb adenocarcinoma, not otherwise specified. Cervical intraepithelial neoplasia (CIN) was also present in 4 AIS patients (CIN2 in 1 and CIN3 in 3) and 1 clear cell adenocarcinoma patient (CIN3) (**Table 1**).

HPV-ISH

HPV-ISH produced positive signals in 17 (63%) of AIS and CA tissues assayed (**Table 1**, **Figures 1** and **2**). On correlation with the histological results, 7 (58%) mucinous adenocarcinoma, 6

(86%) AIS, 2 (100%) serous adenocarcinoma, 1 (100%) adenosquamous carcinoma, and 1 (100%) poorly differentiated adenocarcinoma specimens were positive. Positive HPV-ISH signals were not present in the clear cell and endometrioid adenocarcinoma specimens. Diffuse signal was seen in 7 cases (3 AIS, 2 mucinous, 1 serous, 1 poorly differentiated), the dot-like pattern in 4 cases (1 mucinous, 1 AIS, 1 serous, and 1 adenosquamous), and both patterns were observed in the remaining 6 cases (4 mucinous and 2 AIS). There was no significant correlation between the ISH staining pattern and histological type. In 3 cases (2 serous and 1 mucinous), ISH was performed on metastatic lymph node lesions which displayed the same positive HPV-ISH signal patterns as the primary tumors (**Figure 3**).

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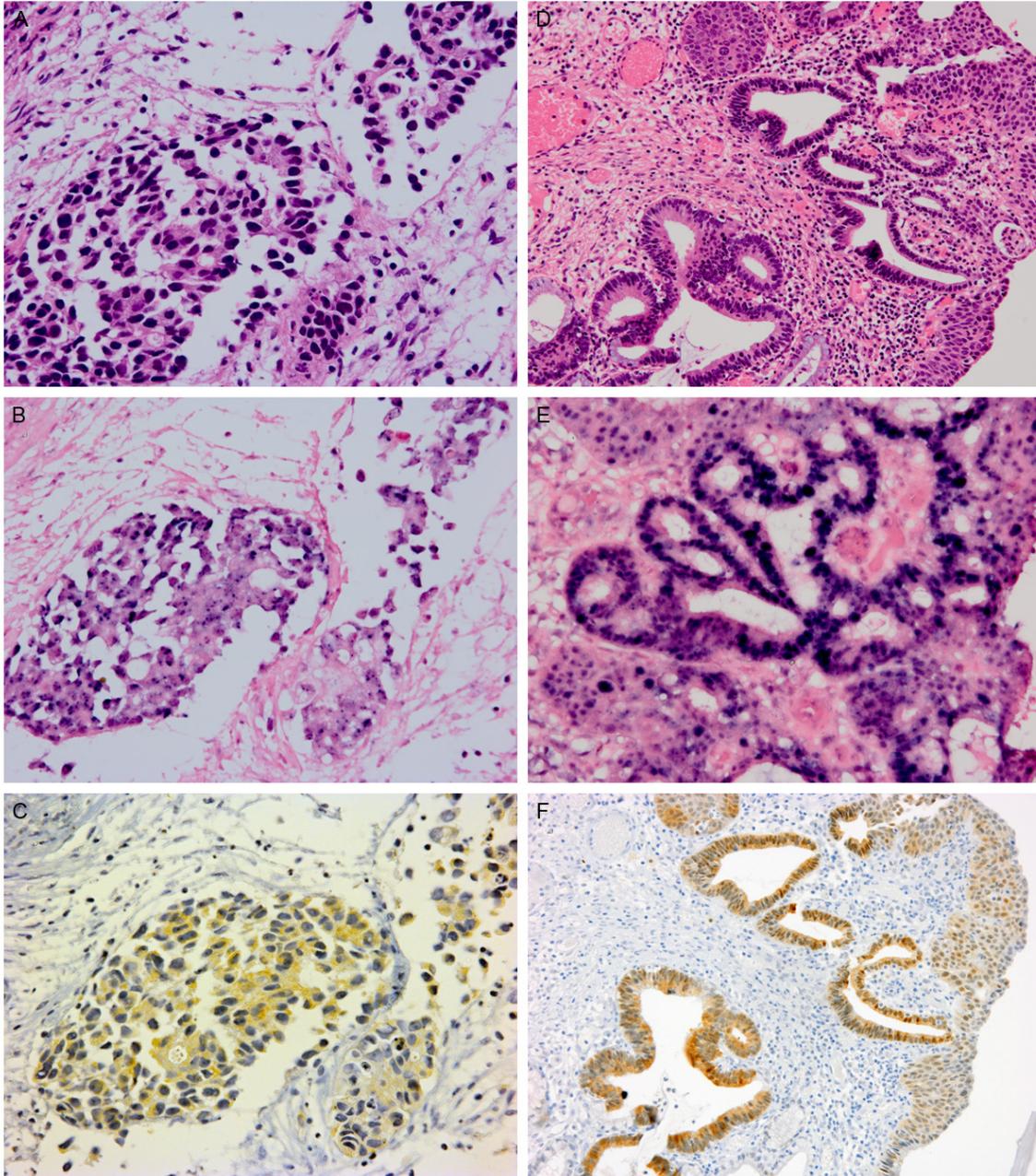


Figure 1. A: A mucinous adenocarcinoma showing intranuclear, dot-like signals indicating in situ hybridization (ISH) detection of HPV (B). C: Immunohistochemical staining indicates that the tumor cells are diffusely stained with p16. D: An adenocarcinoma in situ (AIS) with CIN3 showing diffuse HPV-ISH signal in the nuclei of AIS as well as CIN (E). F: AIS and CIN are both positive for p16 staining.

HPV-PCR

HPV-DNA was detected by PCR in 18 cases (67%) (**Table 1**): 9 (75%) mucinous adenocarcinoma, 5 (71%) AIS, 2 (100%) serous adenocarcinoma, 1 (50%) endometrioid adenocarcinoma, and 1 (100%) poorly differentiated adenocarcinoma were positive. All cases of

clear cell adenocarcinoma and adenosquamous carcinoma were negative.

HPV genotyping

HPV-16 was identified in 5 cases (3 mucinous adenocarcinomas and 2 AIS). HPV-18 was detected in 2 cases (1 mucinous adenocarci-

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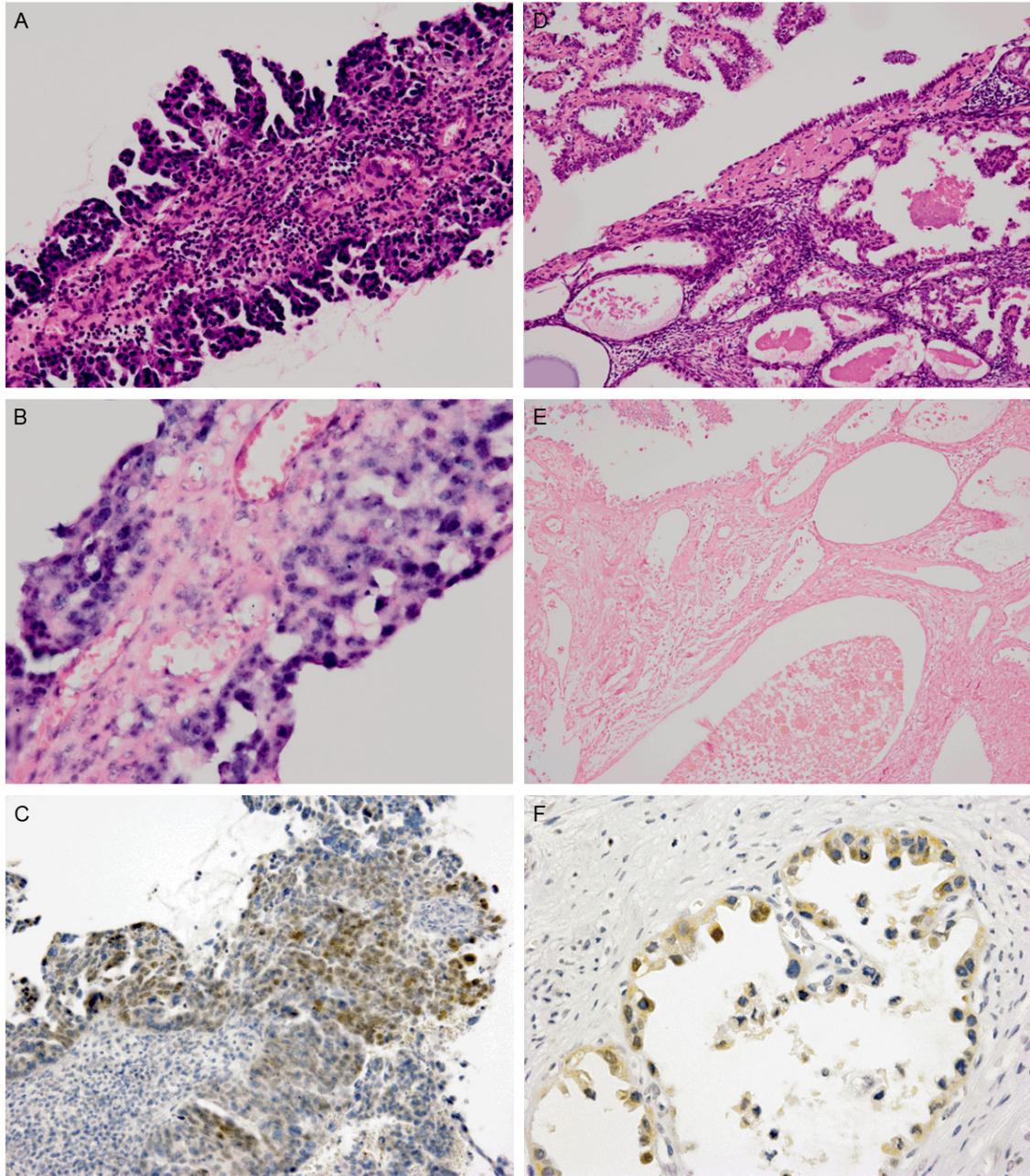


Figure 2. A: A serous adenocarcinoma showing diffuse HPV-ISH signal (B). C: The same tumor with positive p16 staining. D: A clear cell adenocarcinoma negative for HPV-ISH (E), but showing focal staining with p16 (F).

noma and 1 AIS). HPV-45 was identified in 1 mucinous adenocarcinoma. No specific HPV subtypes were identified by this method in the remaining cases (**Table 1**). All HPV-16 positive mucinous adenocarcinoma cases were in stage Ib1, 1 of the HPV-18-positive mucinous adenocarcinoma case and the HPV-45 positive case were in stage IIIb. The ages of the HPV-16 cases ranged from 38 to 78 (mean 54) years, the

ages of the HPV-18 cases were 52 years, and the HPV-45 case was 66 years.

p16 IHC

Immunohistochemical analysis positively identified p16 in 25 cases (93%) (**Table 1, Figures 1 and 2**). Each case of mucinous adenocarcinoma and AIS were p16 negative, and expression

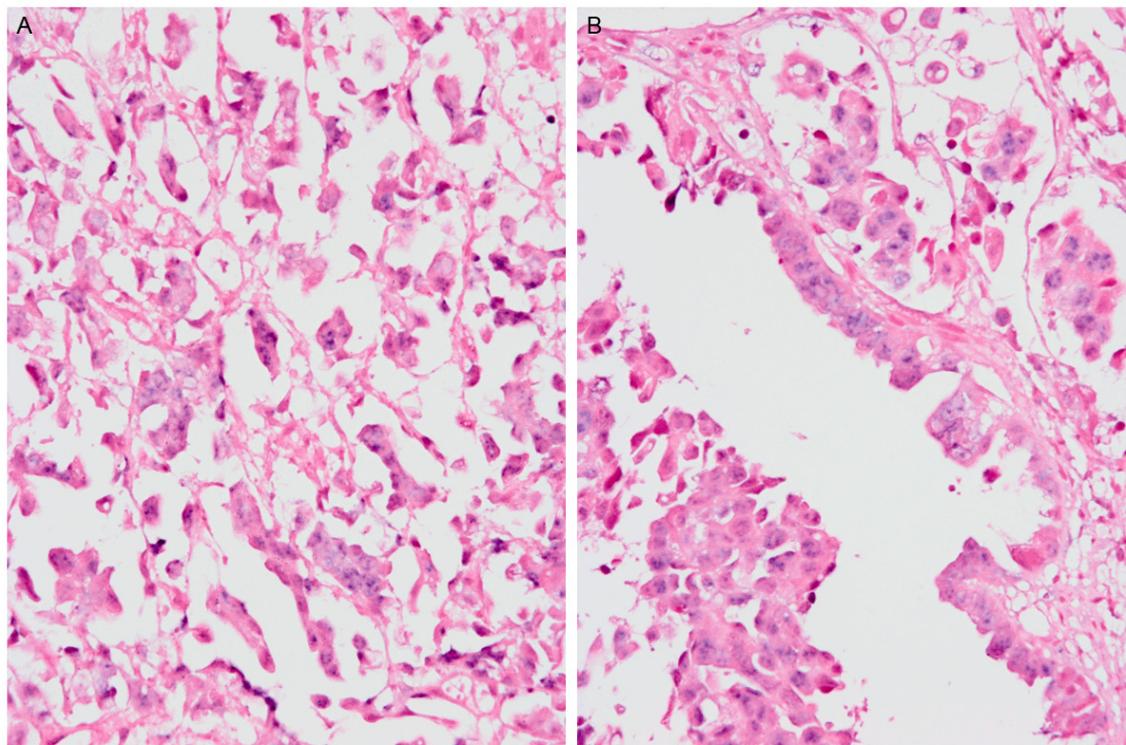


Figure 3. A: A serous adenocarcinoma showing dot-like signals by HPV-ISH. B: A metastatic tumor in a lymph node has the same HPV-ISH signal pattern as the primary tumor.

was focal and weak in the 2 cases of clear cell adenocarcinoma. Interestingly, p16 was positively identified in 4 cases (including the 2 clear cell adenocarcinomas), but HPV results were negative using all 3 HPV assays.

HPV-ISH vs. HPV-PCR and genotyping

There was moderate agreement between the HPV-ISH and HPV-PCR/genotyping results (Kappa value 0.41) with a concordance rate of 74% (Table 2). A total of 5 cases had ISH-negative and PCR-positive results. These cases included 3 mucinous adenocarcinoma, 1 AIS, and 1 endometrioid adenocarcinoma. Of the 3 mucinous adenocarcinoma cases, HPV type 16 or type 18 was detected in 2 cases. In 1 case of AIS and adenosquamous carcinoma, results indicated these specimens were ISH-positive and PCR-negative. The AIS case also displayed CIN3; therefore, HPV infection was highly suspected. However, the specimen was obtained by conization and the area near the AIS lesion had been altered by a thermocoagulation artifact. The thermocoagulative effect may have influenced the results of HPV-DNA analysis by

PCR. In the adenosquamous carcinoma case, only a few dots were observed in each tumor cell by HPV-ISH, indicating a low viral load and possibly explaining the negative HPV-PCR result. One mucinous adenocarcinoma case was positive for HPV-ISH and for HPV-45 by GeneSQUARE assay, but negative by GP5+/GP6+PCR analysis. One AIS case was also HPV-ISH and HPV-18-positive but negative by PCR analysis. Combining the results of HPV-ISH and HPV-PCR/genotyping, 22 cases (81.5%) of AIS and CA were considered to be HPV positive. When the 2 clear cell adenocarcinoma cases (which are generally regarded as HPV negative) were excluded, HPV was detected in 88% of cases in this study.

Discussion

In this study, ISH using a probe targeting multiple high-risk HPV subtypes, PCR and genotyping, as well as p16 IHC were performed on specimens from 27 cases of AIS and CA in the Japanese population. To our knowledge, such a comparative study has been rare and none have included systemic analyses of ISH using INFORM HPV8 to detect HPV DNA.

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Table 2. Correlation between the results of HPV in situ hybridization and PCR or genotyping

		HPV-PCR/genotyping	
		+	-
HPV-ISH	+	15	2
	-	5	5

ISH, in situ hybridization.

A total of 63% of the AIS and CA specimens were HPV-ISH positive in this study. These results confirm that HPV infection occurs in cervical glandular neoplasia with relatively high frequency. HPV DNA was detected in 67% of cases by PCR. Furthermore, 74% of cases were positive for HPV following HPV genotyping analysis. Combining the results of HPV-ISH and HPV-PCR/genotyping, 22 AIS and CA cases (81.5%) were considered HPV positive. When 2 cases clear cell adenocarcinoma, in which HPV is generally considered negative, HPV was detected in 88% of cases of cervical glandular neoplasia. In addition, 3 metastatic lesions also had positive HPV-ISH signals. Although performing PCR would be necessary to obtain more accurate diagnoses of HPV infection, using a sensitive HPV-ISH assay can be a valuable additional tool to detect HPV DNA for diagnosing cervical carcinoma [22]. HPV-ISH is relatively easy to perform, and makes it possible to visualize signal patterns and localization in tissue specimens. For HPV detection in FFPE tissues, the combined use of these techniques may be more sensitive than any single method of detection, and should be considered as the gold standard for HPV detection. p16 positive staining was observed in 93% of the cases; however, 4 cases (16%) were determined to be false positives after considering the combined results of HPV-ISH and HPV-PCR/genotyping. These results suggest that using p16 IHC assays as HPV detection in cervical glandular neoplasia should be interpreted cautiously.

In a previously reported study, Tase et al. detected HPV DNA in 42.5% of FFPE sections from CA cases using ISH with mixed probes [7]. Using RNR-RNA ISH, Milde-Langosch et al. found HPV-16 and -18 E6/E7 oncogene expressions in 62.3% of FFPE sections from CA cases [9]. Our findings were fairly consistent with those of previous studies; 5 cases were ISH-negative and PCR-positive. The variation in reported detection rates may reflect differenc-

es in detection methodologies, case selection, the influence of the fixatives used, or variations in viral load. Significantly higher HPV-positive rates have been detected by use of INFORM HPVIII than INFORM HPVVII [6, 17]. Furthermore, Guo et al. reported that ISH-negative and PCR-positive cervical squamous lesions had significantly lower E2/E6 ratios compared to those shown to be HPV-ISH-positive using INFORM HPVIII [6]. They speculated that low levels of episomal HPV, as a consequence of high viral integration, might be associated with these false-negative results. More sensitive and easier HPV-ISH methods are expected in the near future.

Rates of HPV infection in AIS and CA detected by PCR have been between 42% and 96% (mean 72%) in studies using FFPE tissues [3, 23-29]. In the most recent and largest study thus far, 62% of 760 CA cases analyzed were found to have HPV infection [3]. However, PCR inhibitors in DNA extracted from FFPE tissues can interfere with the ability to detect HPV [30]. One AIS case in the present study was ISH-positive and PCR-negative. This lesion was relatively small and had some artificial changes due to thermocoagulation, which could have influenced the quality of the PCR assay [6, 31]. Using GP5⁺/GP6⁺ and PGMY09/11 consensus primer sets, Chan et al. reported the detection of HPV DNA in cervical cancer tissue in a complementary fashion, and Guo et al. demonstrated a 6% increase in positive HPV-PCR results than with the use of GP5⁺/GP6⁺ primers alone [6, 32]. Moreover, using 3 consensus primer sets, GP5⁺/GP6⁺, PGMY09/11, and CPI/IIG, Baay et al. reported a 15% increase than with the use of GP5⁺/GP6⁺ primers alone to analyze cervical carcinoma cases [33].

In this study, 12 cases were HPV-PCR positive using the general GP5⁺/GP6⁺ method for HPV detection, but negative for typing with GeneSQUARE. However, GeneSQUARE only covers 23 subtypes of HPV. In addition, 2 cases were negative by GP5⁺/GP6⁺PCR, but positive by GeneSQUARE typing: a mucinous adenocarcinoma with HPV-45 infection and an AIS case with HPV-18 infection. The reason for this discrepancy was unclear, but it may be associated with the fact that HPV-18 and -45 viral loads are significantly lower than the corresponding HPV-16 load in cervical cancer [34].

Multiple HPV infections were not observed in this study. This may be due to case selected or to the low viral load of secondary HPV. Furthermore, multiple infections have been considered less common in CA than in squamous cell carcinoma. Zielinski, et al. found multiple subtypes in 6% of CA [27], and Pirog et al. reported 7.6% [28]. These findings suggest that invasive CA growth may be triggered by the actions of a single HPV type, rather than by the synergetic actions of multiple HPVs.

The presence of p16 was demonstrated in 93% of AIS and CA cases, including focal and weak staining of 2 cases of clear cell adenocarcinoma. After excluding 4 p16 positive HPV negative cases as false positives, the sensitivity and specificity are 100% and 40%, respectively. Although p16 IHC is considered superior to HPV-ISH for the detection of high-risk HPV in squamous lesions [17, 35], its utility in CA has not been clearly established [9, 12, 13, 16]. Houghton et al. compared p16 immunoreactivity and linear array HPV genotyping (which covers 37 HPV genotypes), and found that 11 unusual morphological types of CA that were HPV-negative showed p16 immunoreactivity. They concluded that p16 immunoreactivity is not a reliable marker of high-risk HPV in CA. Park et al. also demonstrated that 42% of HPV-negative tumors showed p16 overexpression in unusual adenocarcinoma subtypes. The results of the present study also suggest that p16 expression is not specific for the presence of HPV in CA. Retinoblastoma protein (pRb) normally inhibits the transcription of p16; however, p16 overexpression can occur as a consequence of pRb inactivation by the HPV E7 oncoprotein. In some CAs, p16 expression may occur through non-HPV-related mechanisms that also inactivate pRb, but those exact mechanisms have not been elucidated [18]. Another drawback for the use of p16 IHC assays for HPV detection is that there are no standardized criteria for their interpretation. However, p16 analysis may contribute to the early detection of endocervical neoplastic lesions because it was rarely observed in non-neoplastic glandular cells, both in the current and a previous study [36].

In conclusion, a high prevalence of high-risk HPV in AIS and CA is evident from the present as well as recently published studies. For the screening of HPV, combining liquid-based cytol-

ogy and HPV DNA testing for fresh cytological or tissue specimens is currently regarded as the preferred approach [37]. However, the present study indicates that combination of these techniques with additional assays such as HPV-ISH and HPV-PCR/genotyping can also be used to detect HPV with high sensitivity in FFPE tissues from cervical glandular neoplasias. Although their sensitivity and genotype detection can be limited, HPV-ISH is useful for recognizing the distribution of HPV in AIS and CA tissues and may also be used to confirm the cervical origin of metastatic lesions. An investigation of a larger CA population using HPV-ISH analysis will be necessary to measure its usefulness and determine whether prognostic differences related to HPV physical status exist.

Disclosure of conflict of interest

The authors report no conflicts of interest.

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