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Title page

Original Article

Manuscript title: Utility of p16/Ki67 Double Immunocytochemistry in the Detection of Cervical Adenocarcinoma

Running title: p16/Ki67 status in glandular cells

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The authors have no conflicts of interest to declare.

Author Contributions

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Precis for use in the Table of Contents

The CINtec® PLUS Cytology (p16/Ki-67 double immunocytochemistry) can be used as triage for women with a positive human papilloma virus and negative Papanicolaou results, with a higher specificity for the detection of high-grade squamous intraepithelial lesions. This study reveals that CINtec® PLUS Cytology may also be applicable to the detection of cervical adenocarcinoma lesions.

Abstract

Background: Although the incidence of cervical adenocarcinoma has consistently increased, especially among young women, there is no best means for screening. In this study, efficacy of CINtec® PLUS (CINtec; p16 /Ki67 double immunocytochemistry) expression was evaluated in cervical glandular cells.

Methods: Cervical cytology with abnormal glandular cells was examined. CINtec status was analyzed in 100 samples with corresponding surgically resected specimens and 11 samples that exhibited negative results for intraepithelial lesion or malignancy at follow-up. Additionally, 31 negative samples containing benign glandular cells were also included.

Results: Of the 142 samples, CINtec status was diffusely positive in 74, focally positive in 24, and negative in 44. The 74 diffusely positive samples included 70 adenocarcinomas (62 cervical, 7 uterine, and 1 ovarian) and 4 cases of high-grade cervical intraepithelial neoplasia. The 24 focally positive samples included 15 adenocarcinomas (seven cervical, seven uterine, and one fallopian tube) and 9 without malignancy. The 44 negative samples included nine adenocarcinomas (five uterine and four cervical) and 35 without malignancy. The sensitivity, specificity, and positive predictive value, and negative predictive value of the CINtec-diffusely or -focally positive cases for cervical adenocarcinomas were 94.5%, 58.0%, 70.4%, and 90.9%, respectively. In CINtec-diffusely positive cases, these values were 84.9%, 82.6%, 83.8%, and 83.8%, and especially in women aged ≤39 years they were 90.6%, 89.5%, 93.5%, and 85.0%, respectively.

Conclusion: CINtec may support efficient detection of cervical adenocarcinomas.

Keywords: cervical cytology, adenocarcinoma, p16, Ki67, immunocytochemistry

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3) figures: 4

INTRODUCTION

In developed countries, the incidence of cervical adenocarcinoma has consistently increased,[1,2] especially that of adenocarcinoma in situ (AIS), among young women.[3,4] Unfortunately, screening for cervical adenocarcinoma by the Papanicolaou (Pap) test is inconclusive.[5] Persistent high-risk human papillomavirus (HR-HPV) infections are essential for most premalignant and malignant epithelial lesions of the cervix.[6] The HPV test is a reliable method for detecting precancerous lesions with CIN2 or worse, but one of its limitations is that it leads to false positives because transient HPV infection can also be positive.[7] p16INK4A (p16) functions as an inhibitor of cyclin-dependent kinases. When oncogene of HR-HPV is integrated into host cell DNA by the persistence of an HR-HPV infection, retinoblastoma (RB) protein is functionally inactivated and degraded by E7 binding.[8] Since overexpression of p16 is induced by negative feedback of the RB pathway, p16 has been used as a surrogate marker for premalignant and malignant cervical lesions. The immunohistochemistry (IHC) combination of p16 and Ki67, which is a cell growth factor, is widely used for diagnoses of precancerous cervical lesions. CINtec® PLUS Cytology (CINtec; Roche Diagnostics, Tokyo, Japan) is an immunocytochemical cocktail composed of antibodies against p16 and Ki67. CINtec has been reported to be useful as a triage test for HPV-positive women. In the detection of cervical intraepithelial neoplasia (CIN) 2 or CIN3, CINtec shows a higher specificity than HPV tests, specifically, this test has greater accuracy than HPV tests for younger women (≤30 years) who undergo cervical cancer screening.[9-11] However, most of these studies are based on squamous intraepithelial lesions, but only a few reports of the applicability of CINtec for glandular lesions have been published,[12–14] and the sample sizes are very small.[12,13] Among patients who were treated with definitive radiotherapy for stage IIB–IVA cervical cancer, those with adenocarcinoma / adenosquamous carcinoma had a worse prognosis than those with squamous cell carcinoma.[15] However, the 5-year overall survival rate of stage I patients has been reported to be not significantly different for adenocarcinoma and squamous cell carcinoma.[16] Therefore, it is important to detect and treat cervical adenocarcinomas at an earlier stage to improve the prognosis of patients with these tumors. For the efficient detection of cervical adenocarcinomas, we investigated the reactivity of CINtec in benign/atypical/malignant glandular cells of the cervix.

MATERIALS AND METHODS

This study was performed with the approval of the ethics committee of Osaka International Cancer Institute (approval #21040).

Of 12,094 cervical cytological specimens at our institute from October 2017 to June 2021, abnormal glandular cells were detected in 199 specimens, which were categorized as atypical glandular cells, not otherwise specified (AGC-NOS), atypical glandular cells, favor neoplastic (AGC-FN), AIS, or adenocarcinoma according to the 2001 Bethesda System.[17] In this study, atypical endometrial cells were excluded. Since most endometrial cells that appeared in the cervical specimens were observed to be in small groups (usually 5–10 cells per group),[18] the number of cells that were found is small. Therefore, these cells were considered unsuitable for immunocytochemistry. Of 199 specimens, 185 were subjected to CINtec double staining. In 185 samples, 32 were excluded due to insufficient cellularity. Therefore, the CINtec status was evaluated in 153 samples. Of these 153 samples, 100 with corresponding surgically resected specimens (cone or hysterectomy specimens) and 11 without malignancy in histological or cytological follow-up for a minimum of 21 months were included in this study. Moreover, 31 samples that were classified as negative for intraepithelial lesion or malignancy (NILM) with benign glandular cells were also included. The histological diagnoses were based on the WHO Classification of Tumours of Female Reproductive Organs, 4th edition[19] (Table 1). CINtec double staining was performed according to the manufacturer's instructions. The CINtec status was determined by the CINtec double staining criteria shown below.

Specimen preparation

After cytological specimens were obtained from the uterine cervix, the instruments used for sampling were placed in Cellprep® (CP) Cervical/Oral Cavity vials (Roche Diagnostics, Tokyo, Japan). CP vials stored within 5 weeks at 20°C–25°C were used. CP slides for CINtec double staining were manufactured from residual materials in CP vials, using the CP PLUS device, which is semi-automatic. CP slides were postfixed in 95% ethanol and dried for 1–48 h at 20-25°C before the CINtec double stain.

CINtec® PLUS double staining

CINtec double staining was implemented according to the manufacturer's instructions by setting the CINtec® PLUS Cytology Kit in the VENTANA BenchMark GX (Roche Diagnostics, Tokyo, Japan). The staining protocol for CP was similar to the protocol recommended for ThinPrep® cytology samples. The primary antibodies used were monoclonal mouse anti-Human $p16^{INK4a}$, Clone E6H4™, and monoclonal rabbit anti-Human Ki-67 Clone 274-11 AC3. CINtec double staining consists of two distinct colored reaction products. Brown staining of cells

(cytoplasmic and/or nuclei) reveals p16 overexpression, whereas red staining of cells (nuclei) reveals Ki-67 expression. The presence of brown cytoplasmic staining in cervical epithelial cells and prominent red nuclei in the same cells indicates positivity for the CINtec test. The CINtec test was regarded as negative if brown cytoplasmic staining and red nuclear staining did not coexist within the same cells (Fig. 1). Specimens processed according to the same protocol as patient samples served as positive controls. The positive controls used CP specimens with corresponding histologic diagnoses of CIN2 or worse. Each staining run included one positive control slide. Normal squamous epithelial cells to be negative for expression of p16 and Ki-67 antigens were used as internal negative controls. The CINtec results were considered valid only if the positive and negative controls each demonstrated appropriate staining. Although double staining in even a single squamous cell was originally classified as positive, in this study, CINtec positivity was defined as one or more clusters of double-stained cells because the targets were glandular cells.[14] Furthermore, CINtec positivity was subclassified with reference to the subclassification of p16 IHC in the cervical adenocarcinoma.[20,21] Diffuse positivity was defined as clear and uniform double staining of most target cell clusters (≥50%) (Fig. 2). Focal positivity was defined as an insufficient number of double-stained target cell clusters (<50%) (Fig. 3) or five or fewer double-stained clusters on the whole slide. When the intensity of double staining was weak or when a single double-stained positive cell was observed in a cell cluster, they were not counted as positive cell clusters (Fig. 4).

Statistical analysis

The 142 specimens were divided into two groups: CINtec (focal + diffuse) group, and CINtec (diffuse) group. The CINtec (focal + diffuse) group was analyzed as CINtec positive, including focal or diffuse positives. The CINtec (diffuse) group was analyzed with only diffusely positive for CINtec, whereas others were considered negative. Moreover, the evaluation was stratified into patient age ≤39 years, 40–59 years, and ≥60 years. The following analysis was performed by defining adenocarcinoma of the cervix as having a disease and defining others (including other adenocarcinomas) as having no disease. To compare the diagnostic performance in the two groups, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), and negative likelihood ratio (LR−) with 95% confidence intervals (CIs) were calculated. Fisher's exact test was performed to evaluate the usefulness of two groups in detecting cervical adenocarcinoma. P values <.05 (two-sided) were considered statistically significant. To investigate the association between age and CINtec status in the detection of cervical adenocarcinoma, a logistic regression model was used to calculate

odds ratios (ORs) with 95% CIs and P values. All statistical analyses were performed using Easy R (EZR; Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R.[22]

RESULTS

The CINtec statuses are presented in Table 2 and Table 3 and are compared with the cytological and histopathological diagnoses. Of 142 samples, 74, 24, and 44 samples were diffusely positive for CINtec, focally positive, and negative, respectively. Of 24 focally positive samples, 21 had positivity in five or fewer clusters (mean: 2.5 clusters) on the whole slide, and three exhibited partial positivity (<50%) in the target cells. Of the 73 cases diagnosed cervical adenocarcinomas including AIS on corresponding surgic al specimens, 62 were diffusely positive for CINtec, seven were focally positive, and four were negative. Of the 44 samples deemed to be negative for malignancy according to their corresponding surgical specimens or cytopathological follow-up, nine samples were focally positive for CINtec. Of the six mucinous carcinoma, gastric type (GAS) cases, four were negative for CINtec and two were focally positive (Fig. 3). In 21 specimens of carcinomas derived from areas of the female genital tract other than the cervix, eight were diffusely positive for CINtec. The eight histological diagnoses included four cases of serous carcinoma (uterus), one case of serous carcinoma (ovary), one case of endometrioid carcinoma, G1 (uterus), one case of clear cell carcinoma (uterus), and one case of carcinosarcoma (uterus).

Table 4 presents the CINtec status classified by age group. As age increases, the proportion of tumors in areas of the female genital tract other than the cervix to cervical tumors also increased.

Specificity, PPV, and LR+ were highest in the CINtec (diffuse) group in women aged ≤39 years. Sensitivity and NPV were highest in the CINtec (focal + diffuse) group in women aged 40–59 years. No statistically significant difference was observed in the detection of cervical adenocarcinoma in patients over 60 years in each of the two groups (P >.05) (Table 5).

The ORs of each variable (age, CINtec status) for the detection of cervical adenocarcinoma are listed in Table 6. If CINtec status was considered only diffuse positivity as positive, the CINtec diffusely positive status was highly associated with the detection of cervical adenocarcinoma regardless of age, with an OR of 23.80 for CINtec (95%CI: 9.64–58.70, P <.0001). If both diffusely and focally positive CINtec statuses were regarded as positive, age and CINtec were significant independent factors, with an OR of 0.96 for age (95%CI: 0.92–0.99, P =.02) and an OR of 22.20 for CINtec (95%CI: 7.16–68.80, P <.0001).

DISCUSSION

The population of patients with cervical adenocarcinoma has consistently increasing in developed countries, and the diagnosis of cervical adenocarcinoma, especially at earlier stages, poses a significant challenge. Studies of cervical adenocarcinoma have reported that the prevalence of HPV varies depending on the tumor subtype. They showed high positivity among patients with AIS, adenosquamous carcinoma, endocervical adenocarcinoma (usual type), and considerably low positivity in rare subtypes.[23–25] Also, patients with HPV-positive adenocarcinoma were younger than those with HPV-negative adenocarcinoma.[24, 25] Specifically, HPV was found in 89% of women aged <40 years, but only in 43% of women aged >60 years.[26] Thus, it was suggested that the recognition of abnormal glandular cells of the cervix by CINtec in women aged ≤39 years might provide useful information for cervical adenocarcinoma screening.

Few reports have been published on the use of CINtec for glandular lesions. Singh M et al. investigated the diagnostic performance of CINtec and compared it with HR-HPV testing for CIN2/3 and glandular lesions, but the number of cases with glandular lesions (n = 6) was scarce.[12] Yu et al. evaluated CINtec for the detection of precancerous cervical lesions and cancers in China. However, adenocarcinoma accounted for only a small portion (n = 7) of all cases.[13] Ravarino A et al. studied the utility of CINtec for 47 liquid-based cytology (LBC) specimens with histologic diagnosis of AIS or early invasion, and 16 negative samples. They reported that performance of CINtec for AIS (including early invasion) was sensitivity 97.37%, specificity 83.33%, PPV 92.50%, and NPV 93.75%. There was a significant difference in the expression of CINtec between positive and negative cases, but the positive cases were limited to cases of AIS or early invasion.[14] We analyzed the CINtec status of 142 LBC specimens, focusing on the glandular cells encountered in routine clinical practice. This is the first paper to investigate the expression of CINtec, focusing on various glandular lesions that may be encountered. As the result, in CINtec- diffusely positive cases, these values were 84.9%, 82.6%, 83.8%, and 83.8%, especially in women aged ≤39 years, 90.6%, 89.5%, 93.5%, and 85.0%, respectively. Sensitivity and NPV were inferior to the results of Ravarino A et al., because of inclusion of glandular lesions originating from the uterine corpus, and HPV-independent cervical adenocarcinomas. However, it was considered to be sufficiently practical.

AIS lesions, which are precursors to invasive adenocarcinoma, are rather deep and highly localized in the cervical crypt, and thus, these lesions are difficult to recognize.[27] It is also not easy to recognize AIS lesions by

colposcopy. The advantage of the Pap test is that cells present in the lesion can be extensively sampled in cases with uncertain lesions. Krane JF et al. reported that the sensitivity of Papanicolaou stain specimens for cervical adenocarcinoma was 45%, and 76% excluding inadequate specimens. The reason for this diminished sensitivity was a poor recognition of neoplastic cells that resembled a fragment of endometrial cells, tubule metaplasia, or reactive endocervical cells.[5] Pradhan D et al. investigated 3007 histological follow-up cases reported as AGC. Consequently, only 57 cases (1.9%) had endocervical glandular lesions.[28] Therefore, the detection of cervical glandular lesions in the Pap test is considered to be insufficient. In addition, the diagnosis of AGC varies considerably among observers, suggesting the need for continuous education and training.[29] The introduction of objective indicators, such as CINtec, may be useful in reducing interobserver variability.

We evaluated the diagnostic performance of CINtec stratified by patient age ≤39 years, 40–59 years, and ≥60 years. The specificity, PPV and LR+ were highest in the CINtec (diffuse) group in patients aged ≤39 years. These results may reflect an increase in the incidence of endometrial carcinoma with aging. Most patients with endometrioid carcinoma are postmenopausal, as the disease is relatively uncommon in young women. Only 1%–8 % of endometrial carcinomas occur in women aged <40 years. Moreover, patients with serous carcinoma, clear cell carcinoma, or uterine carcinosarcoma are older than women with endometrioid carcinoma.[30] Therefore, in women 39 years and younger, CINtec positivity for endometrial carcinoma has little effect.

When glandular cells of undetermined significance are recognized in a cervical smear, the diffusely CINtec positive status represents a high possibility of cervical adenocarcinoma (OR by logistic regression; 23.80), especially, in women aged ≤ 39 years (LR+; 8.609). In women aged < 60 years, especially in women aged 40-59 years, the CINtec negative status is highly likely not to have cervical adenocarcinoma (LR−; 0.045). Focally and diffusely subclassified CINtec positivity for glandular cells of undetermined significance may serve as a triage for cervical scrutiny.

Of the 44 samples which were not malignant, nine samples showed focal positivity for CINtec. In p16 IHC, staining of glandular epithelium in sporadic single cells was considered negative.[31] Murphy et al. reported that tubal endometrial metaplasia exhibited cytoplasmic staining with occasional nuclear positivity with p16 IHC, and normal fallopian tubes exhibited focal positive cytoplasmic staining.[32] Riethdorf et al. reported that tubal endometrial metaplasia and atypical metaplasia expressed both moderate or high Ki-67 index and moderate to strong p16 staining. However, their distribution was described as heterogeneous compared with AIS.[33] In our

study, nine focally positive samples may reflect such a heterogeneous distribution. We suggest that the assessment of CINtec positivity for glandular cells should be limited to diffuse positivity.

Of the six GAS cases, four were negative and two were focally positive for CINtec. In the study by Carleton C et al., of the 47 cases of cervical and vaginal GAS, 14 cases showed focal positivity for p16 IHC and four showed diffuse immunoreactivity.[20] Significant proportion of cervical adenocarcinomas were p16-positive in the absence of HPV, which suggests that the Rb pathway was rendered dysfunctional by some unknown mechanisms other than HPV infection.[21] Recognition of morphological features is also important for GAS screening.[34]

In 21 specimens of carcinomas derived from areas of the female reproductive tract other than the cervix, eight were diffusely positive for CINtec. Chiesa-Vottero et al. analyzed the expression of p16 IHC in 11 cases of uterine serous carcinoma and 10 cases of ovarian high-grade serous carcinoma. As a result, p16 was diffusely expressed in all 11 uterine specimens and in 5 of 10 ovarian specimens.[35] In our study, only one of five uterine serous carcinomas was focally positive for CINtec, but that case was strongly and diffusely positive for p16 and was partially positive for Ki67. Conversely, endometrioid carcinoma was usually focal positive and occasionally positive for 100% of target cells.[36] Additionally, normal endometrial glands showed varying staining capacity for p16.[28] Furthermore, p16 presented diffuse positivity in 50% of clear cell carcinomas.[37] In uterine carcinosarcomas, p16 IHC shows an almost equally high expression in epithelial (74%) and mesenchymal components (71%).[38] The characteristics of p16 IHC in endometrial cancers should be considered when assessing the CINtec status of cervical cytological specimens.

Based on the above, we propose the following: If glandular cells in the cervical specimen shows diffuse positivity for CINtec, immediate scrutiny of the cervix of women aged ≤39 years and the genitals of women aged ≥40 years is recommended. For focally positive cases, careful follow-up is required.

In conclusion, CINtec may useful for screening of cervical adenocarcinoma in cases where it is difficult to identify atypical glandular cells in cervical specimens. Nevertheless, in women aged ≥40 years, diffuse positivity for CINtec cannot exclude glandular lesions of the female genital tract in areas outside the cervix. HPV-independent cervical adenocarcinomas, which are represented by GAS, may be a pitfall not only with HPV tests and vaccines but also with CINtec.

Limitations

The results of our study are limited by its single-center design. Our institute is intended for patients who require a more detailed examination as a result of cervical cancer screening. Therefore, this test is more targeted than the true screening for cervical adenocarcinoma. Moreover, this study targets histologically confirmed cases with surgically resected specimens to identify the primary lesion, and did not include cases where only a biopsy was performed.

Table 1. Cytological and histological diagnoses of 142 specimens

Histological diagnosis	Cytological category									
	AD	AD + HSIL$^+$	AIS	AIS + HSIL$^+$	AGC-FN	AGC-FN + HSIL$^+$	AGC-NOS	AGC-NOS + HSIL	NILM	Total
Cervix										
UEA	22	1	1	1	1					26
UEA + CIN2$^+$	1	2	2	3	1		2			11
UEA + Small cell carcinoma						1				1
Mucinous carcinoma, NOS + CIN2$^+$		1								1
Adeno-squamous cell carcinoma	1									1
AIS	4		6	1	1	1				13
AIS + CIN2$^+$		2	3	5	1			1		12
GAS	3		2				1			6
Clear cell carcinoma	1									1
SMILE + CIN2$^+$	1									1
CIN2$^+$	1							3		4
Corpus										
Endometrioid carcinoma, G1	4									4
Endometrioid carcinoma, G2	3						1			4
Endometrioid carcinoma, G3	3									3
Serous carcinoma	5									5
Carcinosarcoma	2									2
Clear cell carcinoma	1									1
Ovary										
Serous carcinoma	1									1
Fallopian tube										
Serous carcinoma			1							1
No malignancy					2		11		31	44
Total	53	6	15	10	6	2	15	4	31	142

Abbreviations: UEA, endocervical adenocarcinoma, usual type; CIN, cervical intraepithelial neoplasia; CIN2$^+$, CIN2 or worse; Mucinous carcinoma, NOS, mucinous carcinoma, not otherwise specified; AIS, adenocarcinoma in situ; GAS, mucinous carcinoma, gastric type; SMILE, stratified mucin-producing intraepithelial lesions; AD, adenocarcinoma; HSIL, high-grade squamous intraepithelial lesion; HSIL$^+$, HSIL or atypical squamous cells cannot exclude HSIL or squamous cell carcinoma; AGC-FN, atypical glandular cells, favor neoplastic; AGC-NOS, atypical glandular cells, not otherwise specified; NILM, negative for intraepithelial lesion or malignancy

Table 2. CINtec® PLUS status for each cytological category

	CINtec status			
Cytological category	(+) diffuse	(+) focal	(−)	Total
Adenocarcinoma	34	13	6	53
Adenocarcinoma + HSIL^{+}	6			6
AIS	12	1	2	15
AIS + HSIL^{+}	10			10
AGC-FN	4		2	6
AGC-FN + HSIL^{+}	2			2
AGC-NOS	2	5	8	15
AGC-NOS + HSIL	4			4
NILM		5	26	31
Total	74	24	44	142

Abbreviations: CINtec, CINtec® PLUS cytology; (+) diffuse, diffuse positive; (+) focal, focal positive; AIS, adenocarcinoma in situ; HSIL, high-grade squamous intraepithelial lesion; HSIL^{+}, HSIL or atypical squamous cells cannot exclude HSIL or squamous cell carcinoma; AGC-FN, atypical glandular cells, favor neoplastic; AGC-NOS, atypical glandular cells, not otherwise specified; NILM, negative for intraepithelial lesion or malignancy

Table 3. CINtec® PLUS status for histological diagnoses of corresponding cytological specimens

		CINtec status			
	Histological diagnosis	(+) diffuse	(+) focal	(−)	Total
Cervix	UEA	23	3		26
	UEA + $CIN2^+$	11			11
	UEA + Small cell carcinoma	1			1
	Mucinous carcinoma, NOS + $CIN2^+$	1			1
	Adeno-squamous carcinoma		1		1
	AIS	13			13
	AIS + $CIN2^+$	12			12
	GAS		2	4	6
	Clear cell carcinoma		1		1
	SMILE + $CIN2^+$	1			1
	$CIN2^+$	4			4
Corpus	Endometrioid carcinoma, G1	1	1	2	4
	Endometrioid carcinoma, G2		3	1	4
	Endometrioid carcinoma, G3		1	2	3
	Serous carcinoma	4	1		5
	Carcinosarcoma	1	1		2
	Clear cell carcinoma	1			1
Ovary	Serous carcinoma	1			1
Fallopian tube	Serous carcinoma		1		1
No malignancy			9	35	44
Total		74	24	44	142

Abbreviations: CINtec, CINtec® PLUS cytology; (+) diffuse, diffuse positive; (+) focal, focal positive; UEA, endocervical adenocarcinoma, usual type; CIN, cervical intraepithelial neoplasia; $CIN2^+$, CIN2 or worse; AIS, adenocarcinoma in situ; GAS, mucinous carcinoma, gastric type; SMILE, Stratified mucin-producing intraepithelial lesions

Table 4. CINtec® PLUS status classified by age group

Tumor origin	CINtec status			Total
	(+) diffuse	(+) focal	(−)	
Age ≤39 y (n = 51)				
Cervix	31※	2	1	34
Corpus		2		2
No malignancy		4	11	15
Age 40–59 y (n = 75)				
Cervix	35※	3	1	39
Corpus	4	2	2	8
Ovary	1			1
Fallopian tube		1		1
No malignancy		5	21	26
Aged ≥60 y (n = 16)				
Cervix		2	2	4
Corpus	3	3	3	9
No malignancy			3	3
Total	74	24	44	142

Abbreviations: CINtec, CINtec® PLUS cytology; (+) diffuse, diffuse positive; (+) focal, focal positive

※ included two samples of high-grade cervical intraepithelial neoplasia

Table 5. Diagnostic performance of CINtec® PLUS for adenocarcinoma of cervical origin

	Sensitivity		Specificity		PPV % (95%CI)	NPV % (95%CI)	LR+ (95%CI)	LR− (95%CI)	p value
	n/N	% (95%CI)	n/N	% (95%CI)					
All ages (n = 142)									
CINtec (focal + diffuse)	69/73	94.5 (86.6–98.5)	40/69	58.0 (45.5–69.8)	70.4 (60.3–79.2)	90.9 (78.3–97.5)	2.249 (1.695–2.983)	0.095 (0.036–0.250)	<0.001※
CINtec (diffuse)	62/73	84.9 (74.6–92.2)	57/69	82.6 (71.6–90.7)	83.8 (73.4–91.3)	83.8 (72.9–91.6)	4.884 (2.894–8.241)	0.182 (0.105–0.318)	<0.001※
Age ≤39 y (n = 51)									
CINtec (focal + diffuse)	31/32	96.9 (83.8–99.9)	11/19	57.9 (33.5–79.7)	79.5 (63.5–90.7)	91.7 (61.5–99.8)	2.301 (1.353–3.912)	0.054 (0.008–0.386)	<0.001※
CINtec (diffuse)	29/32	90.6 (75.0–98.0)	17/19	89.5 (66.9–98.7)	93.5 (78.6–99.2)	85.0 (62.1–96.8)	8.609 (2.310–32.089)	0.105 (0.035–0.311)	<0.001※
Age 40–59 y (n = 75)									
CINtec (focal + diffuse)	36/37	97.3 (85.8–99.9)	23/38	60.5 (43.4–76.0)	70.6 (56.2–82.5)	95.8 (78.9–99.9)	2.465 (1.657–3.667)	0.045 (0.006–0.314)	<0.001※
CINtec (diffuse)	33/37	89.2 (74.6–97.0)	31/38	81.6 (65.7–92.3)	82.5 (67.2–92.7)	88.6 (73.3–96.8)	4.842 (2.457–9.542)	0.133 (0.052–0.338)	<0.001※
Age ≥60 y (n = 16)									
CINtec (focal + diffuse)	2/4	50.0 (6.8–93.2)	6/12	50.0 (21.1–78.9)	25.0 (3.2–65.1)	75.0 (34.9–96.8)	1.000 (0.323–3.101)	1.000 (0.323–3.101)	1.000
CINtec (diffuse)	0/4	0.0 (0.0–71.6)	9/12	75.0 (42.8–94.5)	0.0 (0.0–80.6)	69.2 (38.6–90.9)	0.000 (0.000–NA)	1.333 (0.962–1.848)	0.529

Abbreviations: CINtec, CINtec® PLUS cytology; focal, focal positive; diffuse, diffuse positive; PPV, positive predictive value; NPV, negative predictive value; LR + , positive likelihood ratio; LR−, negative likelihood ratio; 95%CI, 95%confidence interval

※ $p < 0.05$ statistically significant

Table 6. Effect of age and CINtec® PLUS on the detection of cervical adenocarcinoma calculated by logistic regression

	Odds ratio	95%CI	p value
age	0.97	0.93–1.01	0.17
CINtec (diffuse)	23.80	9.64–58.70	<0.0001※※

	Odds ratio	95％CI	p value
age	0.96	0.92–0.99	0.02※
CINtec (focal + diffuse)	22.20	7.16–68.80	<0.0001※※

Abbreviations: CINtec, CINtec® PLUS cytology; diffuse, diffuse positive; focal, focal positive; 95% CI, 95% confidence interval

※ $p < 0.05$ statistically significant; ※※ $p < 0.0001$ statistically significant

Figure Legends

Figure 1. (A) Cell cluster showing diffuse positivity for CINtec® PLUS. Brown cytoplasmic staining (p16 overexpression) and prominent red nuclei (Ki-67 expression) clearly coexisted in the same cells. A; a case of cervical adenocarcinoma, usual type. (B–D) Cell clusters that were negative for CINtec® PLUS. C demonstrated only p16 staining, D demonstrated only Ki67 staining. The CINtec® PLUS was considered negative in B–D because of a lack of concomitant brown cytoplasmic staining and red nuclear staining within the same cells. B and C; cases without malignancy on follow-up. D; a case of uterine endometrioid adenocarcinoma. (CINtec® PLUS; p16/Ki67 dual staining, original magnification ×60 (A–D))

Figure 2. Diffuse positivity for CINtec® PLUS. (A) Most cell clusters demonstrated diffuse double staining for p16 and Ki67. (B) Atypical glandular cells were stained with two colors, red and brown, in the same cell. (C)Atypical glandular cells with fine granular chromatin exhibited crowding. A–C; a case of cervical adenocarcinoma, usual type. (CINtec® PLUS; p16/Ki67 dual staining, original magnification ×10 (A), ×40 (B), Papanicolaou ×60 (C))

Figure 3. Focal positivity for CINtec® PLUS. (A) Most of the cells exhibited only Ki67 staining. (B) Cell clusters with prominent red nuclei were observed. (C) A small number of cell clusters stained with two colors, red and brown, were noted on the slide. (D) Glandular cells with prominent nucleoli and vesicular chromatin were observed. A–D; a case of mucinous carcinoma, gastric type. (CINtec® PLUS; p16/Ki67 dual staining, original magnification ×10 (A), ×40 (B) (C), Papanicolaou ×60 (D))

Figure 4. Cell clusters which were not counted in the number of positive cell clusters on CINtec® PLUS in this study. (A) The cell cluster is clearly stained for Ki67, but p16 staining is ambiguous. (B) Single double-stained positive cell is located within the cell cluster. Most cells in the cell cluster are stained only for p16. (CINtec® PLUS; p16/Ki67 dual staining, original magnification ×60 (A) (B))