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Pathogenesis of Combined High-Grade Squamous Intraepithelial Lesion and Adenocarcinoma *in Situ* of the Uterine Cervix: Human Papillomavirus Genotype and Methylation Status and Immunohistochemical Study

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ABSTRACT

To determine the etiology of combined high-grade squamous intraepithelial lesion (HSIL) and adenocarcinoma *in situ* (AIS) of the uterine cervix, we examined human papillomavirus (HPV) subtypes, methylation status of the HPV-16 L1 gene, and immunohistochemical staining pattern of Krt7 in 8 cases of combined HSIL and AIS. Overall, 6 (75%) of 8 patients with combined HSIL and AIS were infected by the same subtype of HPV in both HSIL and AIS (cases 1–5, HPV-16; and case 6, HPV-18), whereas 2 (25%) patients showed infection with different subtypes of HPV (case 7, HPV-31 and -18; and case 8, HPV-52 and -16, in HSIL and AIS, respectively). The degrees of methylation at CpG islands within the HPV-16 L1 gene were almost equivalent between HSIL and AIS in cases 1–4, whereas a great difference in CpG methylation patterns between two was seen in only 1 case (case 5). In addition, both patients infected with different subtypes of HPV between HSIL and AIS were positive for Krt7 only within the AIS component. Based on these results, we propose two distinct developmental pathways of combined HSIL and AIS of the uterine cervix, the common pathway and the individual pathway.

INTRODUCTION

Both high-grade squamous intraepithelial lesion (HSIL) and adenocarcinoma *in situ* (AIS) are human papillomavirus (HPV)-associated precancerous lesions of the uterine cervix. Although the prevalence of AIS is considerably less common than HSIL, over half of AIS cases are detected with coexistent HSIL or invasive squamous cell carcinoma^{5, 6}. For the last few decades, several theories have developed about the origin of combined HSIL and AIS lesions; some previous studies reported a difference in HPV genotypes² and immunohistochemical staining patterns between HSIL with and without AIS^{16, 26}. These studies suggest that combined HSIL and AIS lesions are etiologically different from HSILs without coexistent glandular lesions; however, there is little molecular evidence of the carcinogenesis of HSIL and AIS.

In our previous study, we revealed that the frequency of HPV L1 gene methylation is closely correlated with the tumour progression status from precursor lesion to invasive squamous cell carcinoma²¹. High-risk genomic integration of HPV into the infected host cell genome, so-called transforming infection, is considered a key event in the progression of cervical neoplasia through increased E6 and E7 oncoprotein expression^{13, 20, 31}. The host cell alterations observed in HPV-associated cervical cancers include hypermethylation of host cell deoxyribonucleic acid (DNA) resulting in the silencing of the promoter region of tumour suppressor genes. In addition to host cell DNA, the HPV DNA also becomes methylated during carcinogenesis^{4, 8, 19, 32}. In particular, within the entire HPV genome, the regions of the L1, L2, and E5 genes of HPV-16 are particularly susceptible to methylation,

and the methylation frequency of HPV-16 DNA progressively increases as the lesion status changes from benign, precancerous lesions, to malignant^{3, 8, 15}. HPV DNA methylation status has been proposed as a potential biomarker for early diagnosis and improved prognosis of cervical cancer^{25, 33}.

Recently, Herfs et al. reported that immunohistochemical staining with an anti-cytokeratin 7 (Krt7) primary antibody (clone RCK105; Thermo Scientific) highlights a discrete population of cuboidal cells at the squamocolumnar (SC) junction of the uterine cervix, and that these cells may be responsible for most HPV-associated cervical carcinomas regardless of their histology^{10, 11}. According to their report, cervical carcinoma and its precursors originated from the discrete population of SC junction cells also positive for Krt7, reflecting their putative origin.

In this study, we examined HPV subtypes, the methylation status of the HPV-16 L1 gene and the Krt7 immunophenotype in combined HSIL and AIS lesions to compare the carcinogenic pathway and origin of its two components. The aim of this study was to demonstrate the uniqueness of combined HSIL and AIS lesions through the identification of similarities and differences between the HSIL and AIS components of combined lesions.

MATERIALS AND METHODS

Tissue samples and immunohistochemistry

A total of eight combined HSIL and AIS cases were evaluated in this study. Formalin-fixed and paraffin-embedded uterine cervical specimens were removed surgically at Kobe University Hospital (Kobe, Japan) and Hyogo Cancer Center (Akashi, Japan). Institutional review board approval was obtained for this study (Kobe University #1191). Immunohistochemical analysis of p16^{INK4a} (CINtec Histology Kit, clone E6H4TM, mtm laboratories AG, Heidelberg, Germany) and Krt7 (clone RCK105, 1:10 dilution; Thermo Scientific, Waltham, MA) expression was performed with a 3-step immunoperoxidase technique using the Ventana Nexes Autostainer (Ventana Medical Systems Inc., Tucson, AZ).

HPV genotyping

In each case, AIS and HSIL were individually isolated and collected in a 1.5 mL tube through the micro-dissection of ten 10 μ m-thick deparaffinized tissue sections. Dissected tissues were solubilized in lysis buffer (1.0% SDS and 0.1 M NaOH) before incubation at 95°C for 20 min. After centrifugation, the supernatant was purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). HPV typing was determined by performing polymerase chain reaction (PCR) using type-specific HPV primers for HPV-16, -18, -31, -52, and -58 and Takara ExTaq HS polymerase (TaKaRa, Ohtsu, Japan) with 2 μ L genomic DNA extracted from clinical samples in a 15 μ L final reaction volume. The PCR amplification conditions were as follows: preheating for 4.5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. HPV sequence alignment and genotype-specific primer design for HPV-16, -18, -31, -52, and -58 was performed as previously reported²¹.

Bisulphite treatment

Bisulphite treatment was administered as previously reported with some modifications for patients positive for HPV-16 in both HSIL and AIS. Genomic DNA extraction was performed as noted previously and a 5 M bisulphite solution was added to the DNA to convert cytosine to uracil³⁵. After 40 min at 80°C, the bisulphite-treated genomic DNA was purified by using the QIAquick PCR Purification kit (Qiagen). The bisulphite modification was completed by treatment with 0.3 M NaOH for 5 min, after which the bisulphite-treated DNA was extracted by performing gel filtration (GE Healthcare, Princeton, NJ) and used as the PCR template. To confirm the complete bisulphite conversion of the DNA sample, multiple non-CpG cytosines present in our targets were evaluated as the internal control for bisulphite conversion. We used only the sequencing results in which the converted cytosines accounted for >98.0% of the total non-CpG cytosines in each target.

Bisulphite-modified DNA sequencing

To confirm the methylation status of HPV-16 genomic DNA, the L1 gene and long control region (LCR) of HPV-16 were modified by performing bisulphite treatment followed by PCR amplification with type-specific HPV primers. PCR amplifications were performed by using the Takara ExTaq HS polymerase with 2- μ L bisulphite-treated genomic DNA in a 15- μ L final reaction volume. The PCR amplification conditions were as follows: preheating for 4.5 min at 95°C followed by 45 cycles of 30 s at 95°C, 30 s at 52 or 54°C, and 40 s at 72°C. Primer design was performed as previously reported²¹. The PCR-amplified fragments were subcloned into PCR2.1-TOPO vectors (Invitrogen, San Diego, CA). Six to eight cloned DNAs from each tissue were sequenced

by performing Big Dye terminator v3.1 Cycle Sequencing (Applied Biosystems, Foster City, CA) and the methylation ratios of the samples were determined. To analyse L1 gene methylation variation in specimens, we calculated the methylation ratio of the L1 gene (L1MR), which was previously reported as a method to evaluate L1 gene methylation variation [6]. L1MR was calculated using the following formula: $L1MR (\%) = (\text{number of methylated CpGs}) / (\text{total number of CpGs}) \times 100$.

Statistical analyses

The methylation status within HSIL and AIS samples was compared using Fisher's exact test. All differences were considered significant at $p < 0.05$. All statistical analyses were performed with JMP software, version 6 (SAS Institute Inc., Cary, NC).

RESULTS

Clinical characteristics

The clinical data are summarized in Table I. Case 1, 3, 5, and 6 were from Hyogo Cancer Center and Case 2, 4, 7, and 8 were from Kobe University Hospital. The median age at surgery was 42 (range 33–63) years. Reproductive history was available in 6 cases (cases 2–4 and 6–8) and all 6 patients had at least one gravidity and one parturition. In case 2, the patient was 10 years away from menopause, and, in case 4, the patient was in the perimenopausal period. The lesion was detected by performing a Pap smear test during pregnancy in case 7. A preoperative biopsy was performed for every case, and four (50%) of the patients were diagnosed with HSIL and three (38%) with AIS. In the remaining case, there was no evidence of malignancy in the biopsy specimens, but the cervical smear cytology indicated HSIL. As an initial surgery, conisation was performed in six (75%) cases, followed by hysterectomy. Secondary surgeries were performed when positive or close surgical margins were observed 1 week to 3 months after the initial operation. In the remaining two (25%) cases, hysterectomy and bilateral salpingo-oophorectomy were chosen initially. The follow-up period ranged from 10 to 122 months (median, 41 months). Thus far, all 8 patients have no evidence of disease.

Table I. Clinical characteristics of combined HSIL and AIS lesions (n = 8)

Case	Age (years)	Preoperative pathological diagnosis	Initial operation	Additional operation	Follow-up period (months)
1	43	HSIL	Conisation	TAH	15
2	63	HSIL	TAH+BSO	-	10
3	33	AIS	TAH+BSO	-	17
4	53	HSIL	Conisation	TAH+BSO	122
5	36	AIS	Conisation	TAH	40
6	34	HSIL	Conisation	TAH	16
7	39	HSIL	Conisation	TAH	17
8	33	AIS	Conisation	TAH	91

Abbreviations: AIS, adenocarcinoma *in situ*; BSO, bilateral salpingo-oophorectomy; TAH, total abdominal hysterectomy

HPV DNA Detection and Typing

After extraction of DNA from formalin-fixed and paraffin-embedded tissues, HPV subtypes were determined in both HSIL and AIS for all 8 cases. Results are summarized in Table II. With respect to HPV subtype, six (75%) showed the same subtype of HPV in both HSIL and AIS: cases 1–5, HPV-16 (62.5%); and case 6, HPV-18, (12.5%). Two (25%) cases had different subtypes of HPV between HSIL and AIS: case 7, HPV-52 and HPV-16; and case 8, HPV-31 and HPV-18, in HSIL and AIS, respectively.

Methylation Status

The methylation pattern of the 5 cases of combined HSIL and AIS, which contained HPV-16 in both HSIL and AIS, are summarized in Figure 1. We observed various CpG methylation patterns in the L1 gene; however, the mean L1MR of HSIL and AIS was 30.56 ± 10.56 and 34.50 ± 4.33 , respectively, and there was no significant difference between these epithelial lesions ($p = 0.743$; Table II). The frequency of methylated CpGs (meCpGs) in cases 3 and 4 was higher than that in cases 1 and 2, and the incidence of methylation at CpG sites was almost equivalent between HSIL and AIS in cases 1–4. Interestingly, in case 5, a moderate frequency of meCpG was detected in the AIS lesion (L1MR = 24.5%), which was significantly higher than that in HSIL (L1MR = 4.2%).

PATHOGENESIS OF COMBINED HSIL AND AIS LESIONS

Table II. The results of HPV genotyping, HPV-16 L1 gene methylation status evaluation, and immunohistochemical analyses

Case	HPV genotype		L1MR (%)		p16 ^{INK4a}		Krt7	
	HSIL	AIS	HSIL	AIS	HSIL	AIS	HSIL	AIS
1	16	16	13.1	24.3	+	+	+	+
2	16	16	26.6	39.7	+	NA	+	NA
3	16	16	60.2	38.0	+	+	+	+
4	16	16	48.7	46.0	+	+	+	+
5	16	16	4.2	24.5	+	+	+	+
6	18	18	NA	NA	+	+	-	+
7	52	18	NA	NA	+	+	-	+
8	31	16	NA	NA	+	+	-	+

Abbreviations: AIS, adenocarcinoma *in situ*; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; L1MR, L1 gene methylation ratio; NA, data not available

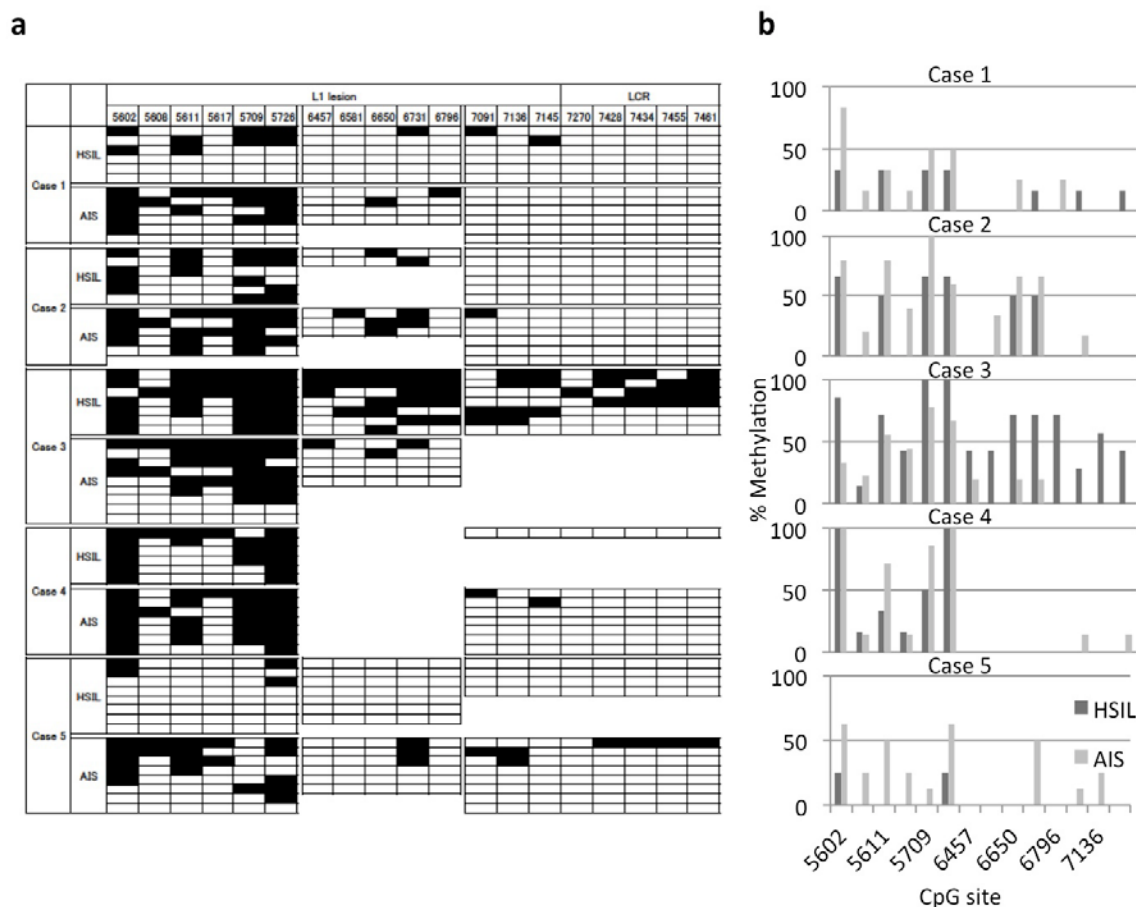


Figure 1. CpG methylation of the L1 gene and the long control region (LCR) in HPV-16. The methylation status of individual CpGs in the L1 gene and the LCR was visualized with bisulphite treatment and amplified by polymerase chain reaction (PCR) with specific primers for HPV-16. a) Methylation pattern of 5 cases of HPV-16-infected combined high-grade squamous intraepithelial lesion (HSIL) and adenocarcinoma *in situ* (AIS). Methylated CpGs are shown as black rectangles and unmethylated CpGs as white rectangles. The number at the top of the figure indicates the nucleic acid sequence position of the CpG in the HPV-16 genome. b) CpG methylation of the L1 gene in 5 cases of combined HSIL and AIS.

Immunohistochemistry

Immunohistochemical evaluation was performed in all cases except in the AIS component of case 2. Since case 2 had only a small AIS component, there was none left in the tissue samples for immunohistochemical analysis after DNA extraction.

Both HSIL and AIS showed strong and diffuse positivity for p16^{INK4a} in all examined cases (Figure 2). In cases 1–5, all tested components were positive for Krt7. Although both the HSIL and AIS components contained

HPV-18, in case 6, Krt7 expression was opposite between HSIL and AIS. Interestingly, cases 7 and 8, with different subtypes of HPV between HSIL and AIS, showed unique staining patterns; Krt7 was positive in the AIS component but negative in HSIL.

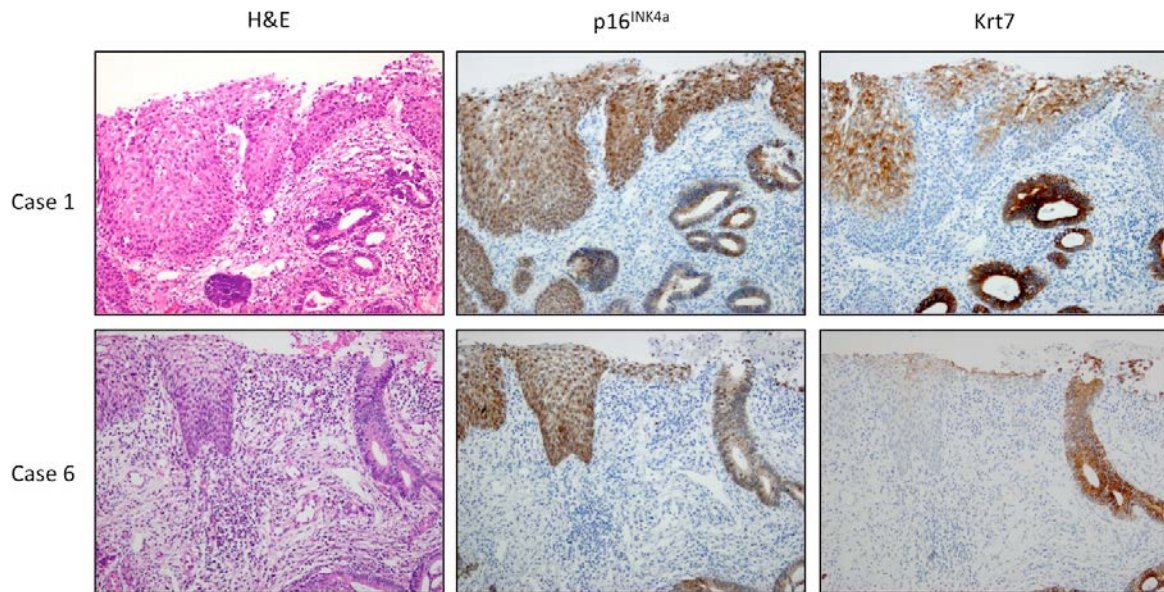


Figure 2. Hematoxylin and eosin staining and immunohistochemistry of combined high-grade squamous intraepithelial lesion (HSIL) and adenocarcinoma *in situ* (AIS). The immunohistochemistry of p16^{INK4a} was diffusely positive for both components in all tested cases. A minority of the cases were positive for Krt7 immunohistochemistry in AIS and negative in HSIL.

DISCUSSION

A number of hypotheses consider that the progenitor cells that develop into HSIL, AIS, and combined HSIL and AIS in the uterine cervix. Previous studies have documented that the reserved cell is the most likely precursor of HSIL^{27, 30}, whereas mature, squamous-type epithelial cells have also been suggested to function as a progenitor cell-type for HSIL³⁴. Moreover, a previous report suggested that the reserved cell is also the progenitor cell not only for AIS²⁸, but also for combined HSIL and AIS^{9, 22}. However, differentiated endocervical cells are also proposed progenitor cells for AIS¹⁵. Very recently, Herfs *et al.*¹⁰ reported a discrete population of SC junction cells with embryonic characteristics that are implicated in the pathogenesis of most HPV-associated cervical squamous cell carcinoma and adenocarcinomas.

According to our data, six (75%) of the AIS lesions that coexisted with HSIL were positive for HPV-16 and the remaining lesions were positive for HPV-18. Although the frequency of the positivity for specific high-risk HPV genotypes such as HPV-16 and HPV-18 in cervical AIS and invasive adenocarcinoma varied between individual studies^{1, 2, 5, 7, 12, 17, 18, 23, 24}, positivity for HPV-16 was more frequently seen in the current study than in previous studies of AIS and invasive adenocarcinoma. Over half of the HSIL components accompanied by AIS lesions were HPV-16-positive in our study, which is a similar frequency to that observed in HSIL and invasive squamous cell carcinoma without glandular lesions²⁹. In terms of HPV infection status, combined HSIL and AIS was different from AIS but similar to HSIL. Our results were slightly different from those of a previous study by Bekkers *et al.*¹⁹ because of the small number of analysed cases; based on the differences in the HPV genotypes among HSIL, AIS, and combined HSIL and AIS, our findings support the theory that combined HSIL and AIS is a distinct type of precancerous cervical lesion. Furthermore, our study revealed that the same HPV subtype is involved in the carcinogenic pathway in six out of eight cases of individual components in combined HSIL and AIS. Surprisingly, each HSIL and AIS component was infected by a different HPV subtype in the remaining two cases.

Among the five cases analysed, the HPV-16 L1 gene methylation pattern was similar between HSIL and AIS components in cases 1–4. This finding indicates that both components generally occur through the same process and express different morphological phenotypes. In other words, both components arise from a single progenitor cell. On the other hand, case 5 showed significant divergence in the HPV-16 L1 gene methylation status between HSIL and AIS. Since the incidence of meCpGs in the HSIL of case 5 was less frequent than that previously reported^{15, 21}, co-infection of other types of high-risk HPV we tested may have played a role in the development of HSIL in this case. We performed further testing for 3 additional subtypes of high-risk HPV (HPV-33, -35, and

-45) in this particular case, however all of them showed negative results (data not shown). Alternatively, the amount of time since the initiation of carcinogenesis might be different between these two components.

Both HSIL and AIS components in all cases showing similar HPV-16 L1 gene methylation patterns (cases 1–4) were positive for Krt7 by immunohistochemical analysis. Two of the combined HSIL and AIS cases that contained different subtypes of HPV showed different Krt7 immunohistochemical staining patterns between HSIL and AIS components (cases 7 and 8). Case 6, which was positive for HPV-18 in both HSIL and AIS components, was also Krt7 positive in AIS but negative in HSIL. In cases 6–8, coexistent HSIL and AIS components showed different immunophenotypes, suggesting that the coexistent lesions had different origins.

In summary, HSIL and AIS in combined HSIL and AIS lesions seem to largely arise from the same progenitor cells located at SC junctions, specifically in a discrete population of Krt7-positive SC junction cells with embryonic characteristics (Figure 3a) and with similar patterns of DNA modifications. However, there are cases of combined HSIL and AIS with different subtypes of HPV, different Krt7 immunohistochemical staining patterns, or significant divergence of HPV-16 L1 gene methylation status between their HSIL and AIS components. This group of cases may arise individually from the different progenitor cells with different HPV infectious backgrounds that collided (Figure 3b). Because Krt7 expression seems to be a common phenotype of cervical cancer regardless of histologic subtype¹⁰, it is no surprise that in case 5, combined HSIL and AIS with significant divergence of the HPV-16 L1 gene methylation status between HSIL and AIS components, is positive for Krt7 in both components. Thus far, there is no single method to discriminate between the two proposed distinctive carcinogenic pathways of combined HSIL and AIS.

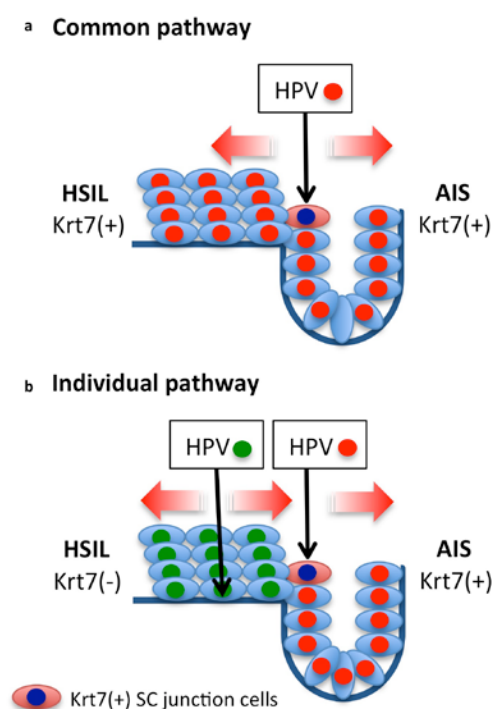


Figure 3. Schematic illustrations of the two distinct proposed pathways leading to the development of combined high-grade squamous intraepithelial lesion and adenocarcinoma *in situ* lesions. a) “Combined” tumour arising from the same progenitor cells located at the squamocolumnar junctions and resulting in two distinct histological phenotypes. b) Collision tumour arising individually with different HPV infectious backgrounds.

In our study, the initial surgical procedure was conisation in 6 out of 8 cases. Since the surgical margins of the uterine corpus side were positive or very close for AIS, all 8 patients were ultimately treated by hysterectomy. Among the 6 patients initially treated with conisation, 4 were preoperatively diagnosed with squamous lesions, and 2 cases were diagnosed with glandular lesions. None of them was diagnosed with squamous and glandular lesions before the initial surgery. Because the unexpected AIS tended to result in a positive surgical margin, combined HSIL and AIS lesions are treated with hysterectomy, whereas most of the HSILs are treated with conisation alone. However, since the number of patients was limited in this study, further clinicopathological, immunohistochemical, and molecular studies will make it possible to distinguish combined HSIL and AIS lesion as distinct precancerous lesions and improve the overall ratio of uterus-conserving treatments.

In conclusion, we described the possible distinct carcinogenic pathways of the combined HSIL and AIS lesion; one is a unique precancerous lesion of the uterine cervix that is etiologically different from pure HSIL or AIS, and the other is a collision neoplasm of independent HSIL and AIS.

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