Uterine cervical squamous cell carcinoma without p16 (CDKN2A) expression: Heterogeneous causes of an unusual immunophenotype

Short running title:

p16-negative uterine cervical SCC

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Abstract

Immunohistochemically p16 (CDKN2A)-negative uterine cervical squamous cell carcinoma (SCC) is uncommon, and there are few reports about its pathological features. This study explored the causes of p16 negativity in such cases. We analyzed diagnostic tissue samples of 5 cases of p16-negative cervical SCC among 107 patients who underwent hysterectomy at Kyoto University Hospital between January 2010 and December 2015. The samples were subjected to immunohistochemical staining, *in situ* hybridization, and a genetic analysis. Two of five cases were positive for HPV by genotyping. One was positive for HPV56 with promoter hypermethylation of *CDKN2A* and co-existing Epstein-Barr virus infection. Another was positive for HPV6 categorized as low-risk HPV with condylomatous morphology. Among the remaining three cases, one had amplification of the L1 gene of HPV with promoter hypermethylation of *CDKN2A* and *TP53* mutation, and one of the other two HPV-negative cases had a homozygous *CDKN2A* deletion, while the other was positive for p53 and CK7. p16-negativity of cervical SCC is often associated with an unusual virus infection status and *CDKN2A* gene abnormality.

Key words: p16, human papilloma virus, uterine cervical squamous cell carcinoma

Introduction

Most uterine cervical squamous cell carcinomas (SCCs) are caused by persistent high-risk human papilloma virus (HPV) infection,[1-3] and the treatment strategy for squamous intraepithelial lesions (SILs) is based on both the histological diagnosis and an HPV typing test. The aberrant overexpression of p16 (*CDKN2A*) is recognized as a characteristic feature of HPV-related cancers among uterine cervical and oropharyngeal SCC.[4, 5] This elevation of the p16 levels in HPV-related cancer and its precursors is caused by the constant inactivation of RB by the E7 protein of high-risk HPV. Loss of the RB function leads to the release of transcription factors, such as E2F1, and results in the overexpression of p16.[6]

Regarding uterine cervix, continuous strong nuclear and cytoplasmic staining for at least one-third of the epithelial thickness suggests HPV-related precancerous lesions,[7] and the use of p16 immunostaining is recommended for distinguishing between cervical dysplasia and its mimics. The frequency of p16 expression in cervical SCC is about 83.5%–100%.[8, 9] However, cervical SCCs that are negative for p16 have not been extensively studied. The histological features and HPV infection status as well as the detection methods applied vary among evaluated lesions, and how SCCs are negative for p16 remains unclear.[10-15]

We investigated the mechanisms underlying p16 negativity by analyzing HPV infection, the *CDKN2A*, and *TP53* mutation status as well as other staining findings (cytokeratin, Epstein-Barr virus [EBV]-encoded small RNA-*in situ* hybridization [EBER-ISH], Ki-67, and p53) for p16-negative uterine cervical SCCs.

Materials and Methods

1. Patient selection of p16-negative uterine cervical SCC

Five cases of p16-negative uterine cervical SCCs were selected by immunohistochemical staining of p16 (CINtec Histology Kit, clone E6H4; Roche-Mtm-Laboratories, Heidelberg, Germany) from among 107 hysterectomy cases treated at Kyoto University Hospital between January 2010 and December 2015. When continuous strong nuclear or nuclear plus cytoplasmic staining of p16 was observed in most tumor cells (over 90% of tumor cells), we labeled the tumor as p16-positive (block-positive). Non-block-positive cases were defined as p16-negative. In well-differentiated, keratinizing cases, p16 positivity was defined as continuous strong staining of the basal cell layer with extension upwards involving at least one-third of the epithelial thickness, according to the Lower Anogenital Squamous Terminology project.[7]

Clinical parameters were retrieved from the clinical records of the five p16-negative cases. Histological parameters, such as the tumor size, histological subtype, lymphovascular invasion, and pathological TNM classification, were also reviewed.

Approval for this retrospective study was obtained from the Institutional Ethics Committee. Patients signed the “Kyoto University Hospital Informed Consent Form

for the Non-therapeutic Use of Histopathological Materials", and the signed forms have now been added to all electronic health records.

2. Immunohistochemical analyses

Immunohistochemical staining was performed using formalin-fixed, paraffin-embedded blocks (FFPE). We used p53 (clone DO-7, 1:100; Dako, Glostrup, Denmark), Ki-67 (clone MIB-1, 1:300; Dako), and CK7 (clone OV-TL12/30, 1:300; Dako) as antibodies, and slides were stained with the Ventana BenchMark ULTRA instrument (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's protocol. Positivity for the nuclear expression of p53 and cytoplasmic expression of CK7 was assessed based on the percentage of positive cells. The Ki-67 index was counted in the hot spot area.

3. *DNA isolation*

DNA was extracted from FFPE. We used the QIAamp DNA FFPE Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

4. Detection of HPV infection

4-1. HPV-DNA genotyping

HPV genotyping was performed for all p16-negative cases as a routine clinical test for HPV detection. HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 68, 70, 71, 73, 82, 84, 90, and CP6108 were examined by the PCR-reverse sequence specific oligonucleotide (PCR-rSSO) method (LSI Medience Corporation, Tokyo, Japan).

4-2. L1 gene detection by consensus primer

In addition, we also used multiple consensus PCR primers sets to detect the L1 gene. The advantage of consensus primers for the HPV L1 gene region is that they can detect a large number of HPVs of both known or unknown types, although they can also cause false negatives because of the loss of the L1 open reading frame during integration of viral DNA into the host genome.[3, 16] Eight primers—My09 (5'-CGTCCMARRGGAWACTGATC-3'), My11 (5'-GCMCAGGGWCATAAYAATGG-3'), Gp5 (5'-TTTGTTACTGTGGTAGATAC-3'), Gp6 (5'-GAAAAATAAACTGTAAATCA-3'), Gp5+ (5'-TTTGTTACTGTGGTAGATACTAC-3'), Gp6+ (5'-GAAAAATAAACTGTAAATCATATTC-3'), Oli-1b (5'-TGYAAATATCCWGATTATWT-3'), and Oli-2i (5'-GTATCIACIACAGTAACAAA-3')—were used. HeLa cells (HPV-positive human

cervical cancer cells) and HMC1 (a human mastocytosis cell line) cells were used as positive and negative controls, respectively. The PCR mix consisted of 1 µL of DNA (cases 2, 4 and 5, positive control, or negative control), 2 µL of each primer pair (My09/My11, Gp5/Gp6, GP5+/Gp6+, and Oli-1b/Oli-2i) and 17 µL of Platinum® Blue PCR Supermix (ThermoFisher Scientific, Waltham, MA, USA). The PCR program was 94 °C for 5 min, (94 °C for 30 s, 45 °C for 30 s, and 72 °C for 60 s) for 65 cycles and 72 °C for 5 min.[17] PCR products were examined as described above.

5. *Analyses of CDKN2A*

5-1. Bisulfite modification and methylation-specific polymerase chain reaction (MSPCR)

DNA methylation patterns in the CpG islands of the *CDKN2A* were detected by the MSPCR technique, as described previously.[18] Bisulfite modification of DNA was performed before MSPCR using the MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (Genetic Signatures, Sydney, Australia) according to the manufacturer's protocol. Then, 4 µL of modified DNA sample (cases 1-5, Hela, or HMC1) and 16 µL of PCR mix (14 µL of Platinum® Blue PCR Supermix and 1 µL of each primer pair) were used for polymerase chain reaction (PCR). The primers were p16-methylated (p16-M) forward (5′-TTATTAGAGGGTGGGGCGGATCGC-3), p16-M

reverse (5′-GACCCCGAACCGCGACCGTAA-3′), p16-unmethylated (p16-U) forward (5′-TTATTAGAGGGTGGGGTGGATTGT-3′), and p16-U reverse (5′-CAACCCCAAACCACAACCATAA-3′). The PCR program for unmethylated primers was 94 °C for 5 min, (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) for 48 cycles and 72 °C for 4 min. The annealing temperature was changed from 58 °C to 67 °C when using methylated primers. PCR products were verified by 2% agarose gel electrophoresis, stained with ethidium bromide, and examined under ultraviolet illumination. We used All-purpose Hi-Lo DNA marker (Bionexus, Oakland, CA, USA) to estimate the band size for electrophoresis.

5-2. Fluorescence *in situ* hybridization (FISH)

We performed dual-color FISH using commercial probes, including the Spectrum Orange-labeled locus specific p16 (9q21) probe and the Spectrum Green-labeled chromosome 9 centromeric probe (Vysis LSI p16 (*CDKN2A*) SpectrumOrange/CEP9 SpectrumGreen Probe; Abbott Molecular, Des Plaines, IL, USA), according to the manufacturer's protocol.

6. Detection of EBV

We assessed the presence of EBV by EBER-ISH, and the EBV latency pattern was determined by the immunohistochemical analysis for latent membrane protein (LMP) 1 and EBV nuclear antigen (EBNA) 2. EBER-ISH (Ventana Medical Systems) was performed with a BOND-III Fully Automated IHC and ISH Stainer (Leica Biosystems, Nussloch, Germany). We used LMP1 (clone CS-1-4, 1:50; Dako) and EBNA2 (clone PE2, 1:30; Dako) with the Ventana BenchMark ULTRA instrument (Ventana Medical Systems) for these assessments.

7. Analysis of TP53 *mutations*

The presence of *TP53* mutations was examined in Exon 4-9 with direct sequencing using FFPE (LSI Medience Corporation). The reference sequence was NG_017013.2. Both the tumor and non-tumor areas of all the five p16-negative cases were examined.

Results

1. Clinicopathological features of p16-negative uterine cervical SCC

Five (4.7%) of 107 cases of uterine cervical SCCs showed non-block positivity for p16. The positivity rates of p16 were 5% (case 1), less than 5% (case 3), and 0% (cases 2, 4 and 5). The clinicopathological characteristics of the p16-negative cases are summarized in Table 1, and representative microscopic images are shown in Figure 1. All patients had a reproductive history. Three of the five p16-negative cases were non-keratinizing SCC (cases 1 to 3), and two were keratinizing SCC (cases 4 and 5). All cases had SILs, and the area was p16-negative, in addition to the invasive area. Case 2 had a sarcomatous component at recurrence. All cases had lymphovascular invasion at the time of surgery.

Case 3 showed an impressive clinical course and morphological features.[19] This patient had a history of cervical intraepithelial neoplasia (CIN) 1-2 and had received a cytological diagnosis of ASC-H (atypical squamous cells, cannot exclude high grade SIL) two years before the definitive diagnosis. She underwent a follow-up cervical biopsy, and the diagnosis was p16-negative CIN3/HSIL. A Loop Electrosurgical Excision Procedure (LEEP) was performed for this lesion. The LEEP specimen had a well-differentiated squamous epithelium with papillary and condylomatous growth pattern and stromal invasion (Figure 2a and 2b). LEEP

specimens showed positive margin, and the followed hysterectomy was performed. The specimen had invasive non-keratinizing SCC beneath the condylomatous component (Figure 2c). p16 was negative both in the condylomatous and non-keratinizing SCC components (Figure 2e and 2i). She died of multiple lung metastasis about two years after hysterectomy.

2. *HPV infection status*

HPV-genotyping by PCR-rSSO revealed that case 1 had HPV56, which is known to be a high-risk HPV; case 3 had HPV6, which is known to be a low-risk HPV; and cases 2, 4, and 5 did not show any HPV infection. Using multiple consensus primer sets, the L1 gene was detected in case 2 by the My09/My11 primer set (Figure 3).

3. *CDKN2A gene status*

To explore the mechanism underlying the p16-negative staining, we examined the status of the *CDKN2A* gene coding p16, including the methylation status and deletion status. Case 1 had a methylated band, and case 2 had both methylated and unmethylated DNA bands. Cases 3 and 4 had unmethylated bands. Case 5 had neither methylated nor unmethylated bands and was not analyzed (Figure 4a). We

found that case 4 had a homozygous deletion of *CDKN2A* according to a dual-color FISH analysis (Figure 4b).

4. Analyses of other factors

Case 1 showed positivity for EBER-ISH and LMP1 (Figure 5a), while EBNA2 was negative. The other four cases were all negative. Case 2 had a nonsense mutation at exon 6 of *TP53* (c.637C>T: p.Arg213Ter) (Figure 5b). The DNA sample of case 5 was not suitable for *TP53* mutation analyses. CK7 was negative in cases 1, 2 and 4 (0%-1%), but focally positive in cases 3 and 5 (30% and 60%, respectively).

Discussion

We found five cases of p16-negative cervical SCCs, and the combination of several mechanisms, including the absence of high-risk HPV infection (cases 3 to 5), the presence of low-risk HPV infection (case 3), the methylation of *CDKN2A* (cases 1 and 2), homozygous deletion of *CDKN2A* (case 4) and EBV infection (case 1), was implied. A *TP53* mutation was found in the case infected by HPV with an unknown genotype (case 2).

Most cervical SCCs are positive for p16, and our search of the literature showed that the frequency of p16-negative cervical SCCs ranged from 0% to 16.5%.[8, 9] In the present study, the frequency was 4.7% (5/107), which was similar to the findings in the previous studies. To our knowledge, there have been 6 reports regarding 16 cases describing the morphology and HPV infection status of p16-negative SCCs (Table 2).[10-15] Some unique morphologies mimicking giant immature condyloma and papillary SIL were reported by Tsai et al. and Liu et al.[12, 15] In our study, we found two unique morphologies in SCCs that included a condylomatous component and sarcomatous component. HPV infection was detected in four of 16 cases (25.0%) in the previous reports, while we found HPV infection in three of five cases (60.0%). The method of detecting HPV has varied, and

the combination of type-specific PCR and multiple consensus primer sets for the L1 gene is ideal for the thorough examination of HPV infection.[17, 20]

One of our cases had low-risk HPV infection. The mechanism behind carcinogenesis with low-risk HPVs is unclear, but some cervical SCCs are reported to have an association with low-risk HPVs.[15, 21] Liu et al. reported three cases of p16 negative HPV6-associated HSIL/SCCs in the uterine cervix.[15] Guimera et al. reported 9 cases of cervical SCCs with low-risk HPVs.[21] In a previous report by Sanjose et al., a single low-risk HPV infection was detected in 0.18% of cervical SCCs (16/8977).[22]

The abnormal status of *CDKN2A* was determined to be one reason for p16 negativity. p16 is encoded by the *CDKN2A* tumor suppressor gene. p16 product inhibits G1–S progression by preventing phosphorylation of the RB protein and preventing activation of transcription factor, E2F1, which leads to an uncontrollable cell cycle. Therefore, loss of the *CDKN2A* function because of promoter hypermethylation or deletion results in RB protein phosphorylation and carcinogenesis.[23] In head and neck SCCs, over half of HPV-negative SCCs were found to have alterations of *CDKN2A*.[24] Regarding the uterine cervix, Nakashima et al. reported that one out of 27 cervical SCCs had hypermethylation of *CDKN2A,* and four out of 35 cervical SCCs had a homozygous deletion of *CDKN2A*.[25] Although the association between the hypermethylation of *CDKN2A* and carcinogenesis is

controversial in uterine cervical SCC,[26-28] in a meta-analysis including 26 studies, its correlation with the pathogenesis was indicated.[29] In our study, hypermethylation of *CDKN2A* was identified in two cases: one with HPV56 infection, and the other with HPV infection of unknown genotype. A homozygous deletion was detected in one case without HPV infection. The association between the methylation status of *CDKN2A*, p16 expression, and HPV infection is inconsistent,[26, 30, 31] and further studies are required.

Co-infection of EBV and HPV in uterine cervical SCCs was also reported.[32, 33] In EBV-associated gastric carcinoma, promoter hypermethylation of various tumor-related genes, including *CDKN2A*, has been identified.[34, 35] In our study of case 1, which had an EBV infection, hypermethylation of *CDKN2A* was noted. Furthermore, in a previous study, Ohtani et al. showed that LMP1 blocks the p16-RB pathway and represses the p16 expression.[36] In our study, case 1 was infected by EBV and showed latency status II (positive for LMP1 and negative for EBNA2). This may be an additional reason why p16 was negative in case 1.

In addition to HPV infection, we also examined the *TP53* mutation status as another etiologic factor. One report found that all cases of HPV-negative SCC had overexpression of p53.[13] In another report, *TP53* mutations were found in 16% of cervical SCCs, with no statistically significant difference in the *TP53* gene mutation

frequency between HPV-positive and HPV-negative samples.[37] In our study, two of five p16-negative SCCs showed p53 overexpression; one case had low-risk HPV, and the other case did not have an HPV infection. In the low-risk HPV case, the overexpression of p53 was focal. Furthermore, one nonsense mutation in *TP53* (c.637C>T: p.Arg213Ter) was detected in the tumor area of case 2. The mutant T peak of codon 637 was relatively low, and there is a possibility that this mutation is subclonal and that background wild-type cells are included. This mutation was previously identified in several reports on malignant tumors of female reproductive organs with sarcomatous component. [38, 39] However, in those previous reports, the p53 immunoexpression showed a null pattern, while that in case 2 in our study showed a wild pattern. Therefore, the utility of p53 expression for diagnosing p16-negative cervical SCCs remains unclear.

We were unable to determine the reason for the negative p16 expression in case 5. The specimen of case 5 was fixed with re-used formalin, which may have resulted in an unstable formalin concentration, as 10% buffered formalin was used in the other 4 cases. Therefore, inappropriate fixation for the genome analysis may have caused our failure to detect HPV infection and methylation of *CDKN2A* in case 5.

The clinical significance of p16-negative cervical SCCs is worth discussing. In CIN, the p16 expression was shown to be not only a diagnostic tool but also a

prognostic factor. p16 block-positive CIN1 cases have a higher tendency to progress to HSIL than p16-negative cases.[40, 41] For CIN2, p16-negative CIN2 shows a higher rate of regression than p16-positive cases, and the p16 expression is an important marker for clinical management.[42] For carcinoma, p16-negative SCCs have a poor prognosis compared to p16-positive cases. In a report by Masoudi et al. on 115 cervical SCC cases, a negative p16 expression was correlated with a lower disease-free survival according to a univariate analysis.[8] Putte et al. studied 220 cervical SCCs and found that a low expression of p16 was significantly related to a decreased overall survival.[43] Our data were too few to compare with p16-positive cases, so a further investigation is required.

Uterine cervical SCCs with low-grade atypia are difficult to diagnose from a biopsy. The differential diagnosis includes a wide spectrum of cervical disease, from non-neoplastic lesions to malignant lesions. Histological findings, such as mild cytological atypia, a low mitotic index, and a low Ki-67 index, are evidence suggesting non-neoplastic lesions.[44] Diffuse block-positive p16 indicates high-risk HPV infection, although p16-negative cervical SCCs do exist. Tsai's report presented a warning case of cervical SCC mimicking immature condyloma without p16 expression.[12] Liu et al. and Guimera et al. also showed impressive findings that p16-negative HPV6-associated cervical SCCs were verruco-papillary lesions on gross and papillary

SIL or well-differentiated morphology according to a histological examination.[15, 21] Our case 3 was similar to these previously reported cases. These findings suggest that cervical SCCs with low-risk HPVs show a well-differentiated morphology and are difficult to diagnose. Liu et al. indicated that nuclear overlap, variable nuclear density throughout the epithelium and anisokaryosis were more frequent in HSIL/carcinoma than LSIL/immature condyloma. In addition, while the Ki-67 expression is seen in over 75% of epithelial cells in carcinomas, its expression is concentrated in the basal third of the epithelium in LSIL/immature condyloma.[15] In such cases, CK7 can be another marker for HPV infection. CK7 contributes to viral episomal replication in high-risk HPV infected cells,[45] and CIN1 with CK7 expression is correlated with CIN2 progression.[46] However, we found no association between the CK7 expression and HPV infection in the present study.

In conclusion, the mechanism underlying the p16-negativity in uterine cervical SCCs was heterogeneous, involving the absence of a high-risk HPV infection, the presence of a low-risk HPV infection, *CDKN2A* gene methylation or deletion, and co-infection of EBV. Another point to be noted concerning p16-negative cases is that a low-grade/condylomatous appearance seen in some cases, which may confound the diagnosis.

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Disclosure Statement

None declared.

Author contributions

MR-K, SM and TK designed the study. MR-K and TK performed the experiments. KA and MM provided clinical information. MR-K and SM wrote the manuscript. HH supervised the study. All authors approved the final manuscript.

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Figure legends

Figure 1. Representative pathological findings of five p16-negative SCCs. (a-e) Cases 1 to 3 were non-keratinizing SCC, and cases 4 and 5 were keratinizing SCC. Case 2 had a sarcomatous component. (f-j) p16 was negative in every case. (k-o) In Case 3, p53 was overexpressed in the hot spot (50%-60%). Case 5 showed p53 overexpression. (p-t) The Ki-67 index of case 5 was low (<5%).

Figure 2. Representative pathological findings of case 3. (a, b) Low-power view of the LEEP specimen. Papillary and condylomatous growth patterns were seen. (c) Low-power view of the hysterectomy specimen. Tumor nests of non-keratinizing SCC (black square**) were observed beneath the condylomatous component (black square*). (d) High magnification of the condylomatous component (*). (h) High magnification of the non-keratinizing SCC (**). (e, i) Both components were negative for p16. (f, j) p53 had wild-type expression in most areas. (g, k) The Ki-67 index was about 30%.

Figure 3. PCR for the L1 gene using multiple consensus primer sets. The L1 gene was detected using the My09/My11 PCR primer set in case 2 (M, Molecular weight marker; Number, Case number; NC., Negative control; PC., Positive control).

Figure 4. An analysis of the *CDKN2A* gene. (a) The methylation status of *CDKN2A* detected by methylation-specific polymerase chain reaction (Lanes 1-5, Case number; Lane 6, Hela; Lane 7, HMC1; M, Molecular weight marker; Lanes u, reactions using p16-U primers specific for the unmethylated CpG sites; Lanes m, reactions using p16-M primers specific for the methylated CpG sites). (b) The homozygous deletion of *CDKN2A* (loss of both orange signals) was observed in case 4. Normal epithelium had two orange and two green (CEP9) signals (inset: normal epithelium).

Figure 5. Inspection results of p16-negative SCCs. (a) EBER-ISH and LMP1 were positive in case 1 (left: EBER-ISH, positive in about 50% of tumor cells, inset: LMP1). (b) The *TP53* mutation (c.637C>T: p.Arg213Ter) in case 2.

Figure 1

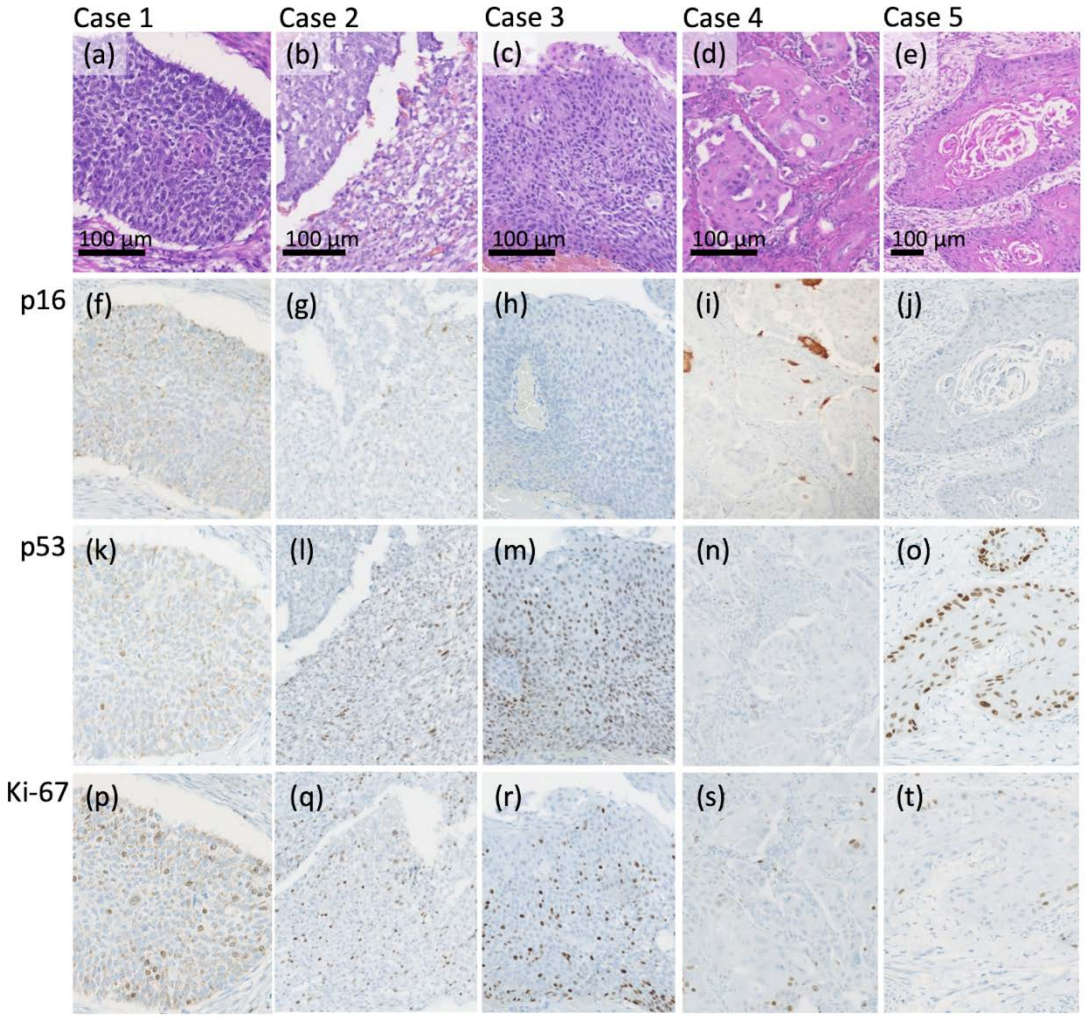

Case 1
Case 2
Case 3
Case 4
Case 5
(a)
(b)
(c)
(d)
(e)
100 µm
100 µm
100 µm
100 µm
100 µm
p16
(f)
(g)
(h)
(i)
(j)
p53
(k)
(l)
(m)
(n)
(o)
Ki-67
(p)
(q)
(r)
(s)
(t)

Figure 2

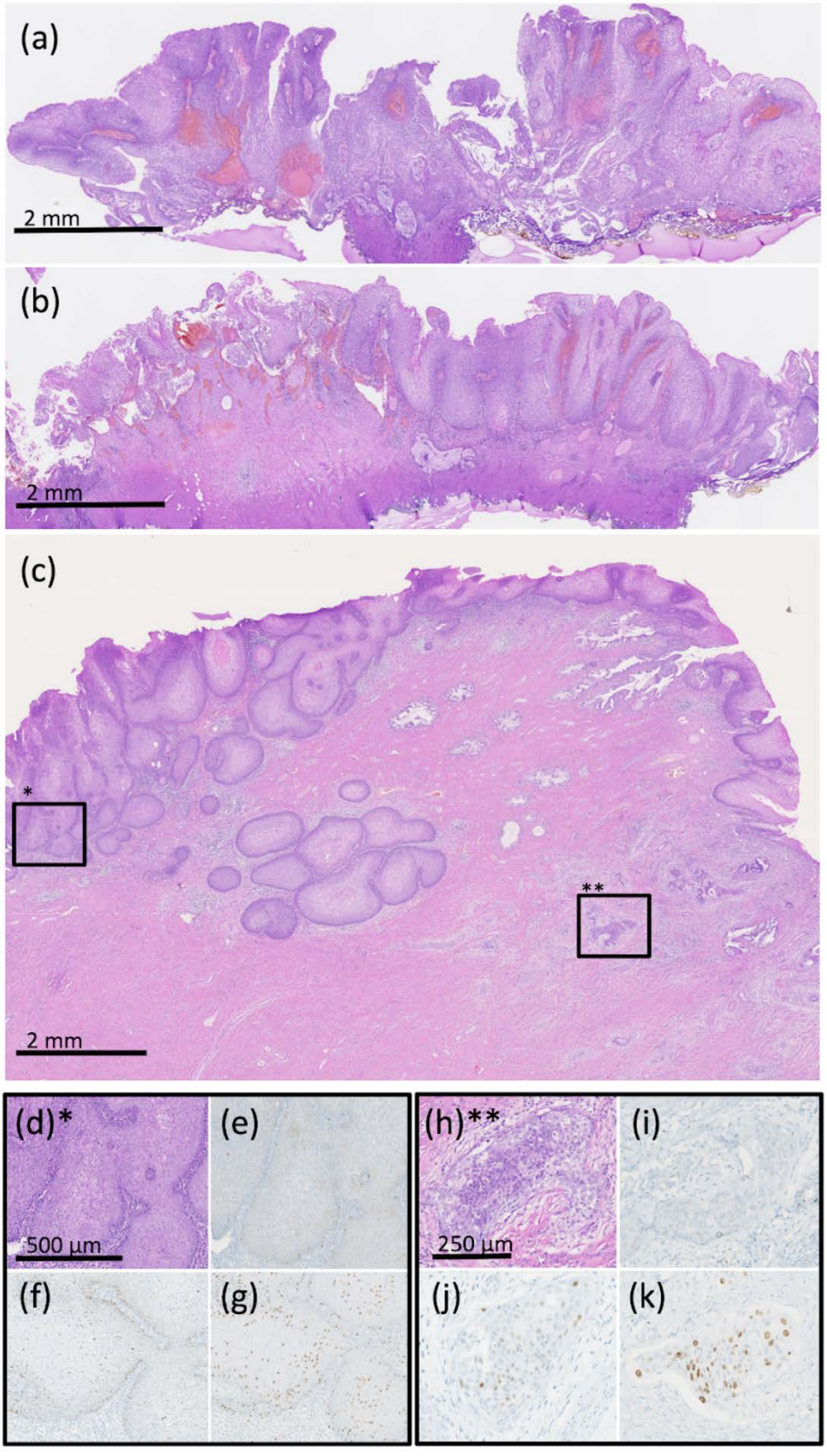


Figure 3

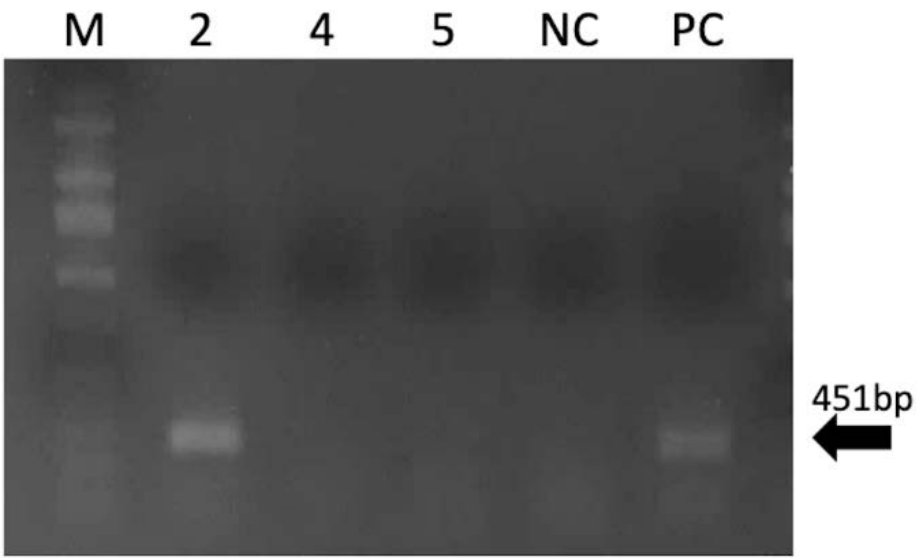


Figure 4

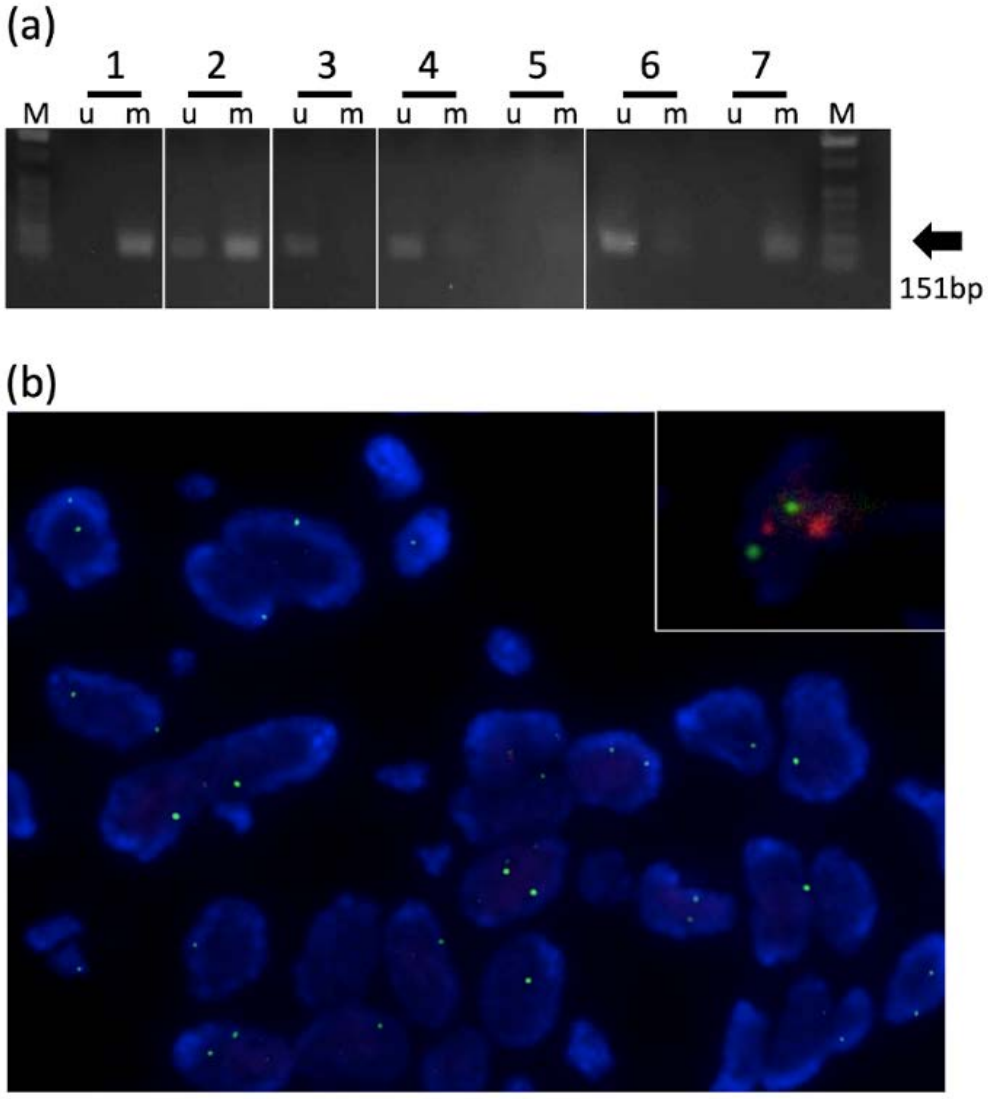
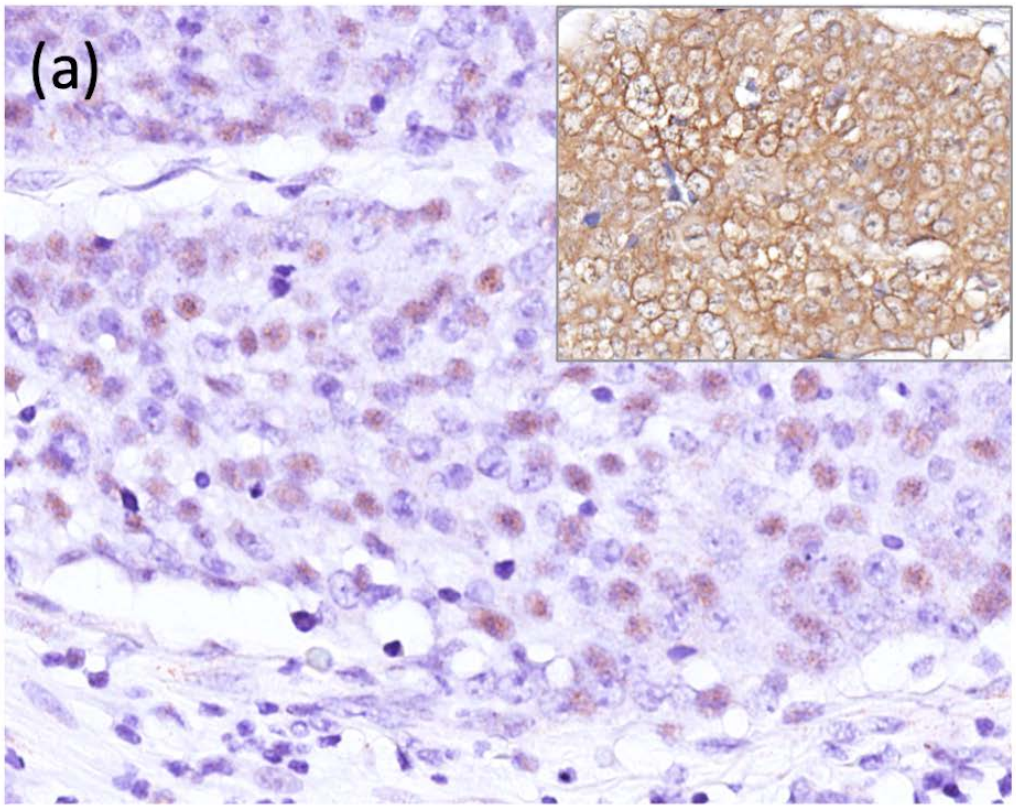
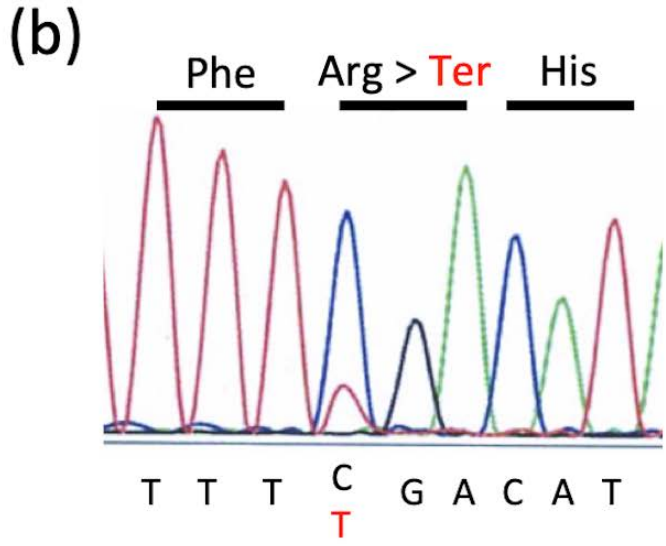


Figure 5





Exon6: c.637C>T: p.Arg213Ter

Table 1. Clinicopathological features of p16-negative cervical squamous cell carcinoma (SCC)

Case number	1	2	3	4	5
Age (years)	54	69	43	65	70
Tumor size (mm)	48	30	33	43	97
pTNM	T2b N1 M0	T1b1 N0 M0	T1b1 N1 M0	T2b N1 M0	T2b N0 M0
Histological subtype	Non-keratinizing	Non-keratinizing	Non-keratinizing Condylomatous	Keratinizing	Keratinizing
HPV					
HPV-L1 (consensus primers)	Not performed	+	Not performed	-	-
HPV genotyping	HPV56 (high risk)	HPV, genotype unknown	HPV6 (low risk)	Not detected	Not detected
CDKN2A (p16)					
IHC	5%	0%	<5%	0%	0%
Promoter hypermethylation	+	+	-	-	Unanalyzable
Deletion	Not performed	-	Not performed	Homozygous deletion	-
EBV					
EBER-ISH/LMP1/EBNA2	+/+/-	-/-/-	-/-/-	-/-/-	-/-/-
p53					
IHC	5%	10%	5-60%	5%	70%
TP53 mutation	-	Exon6 pArg213Ter (c.637C>T)	-	-	Not available
Other IHC					
Ki-67 index	10%	30%	30%	10%	<5%
CK7	1%	0%	30%	0%	60%
Outcome (month)	R.F.	Local recurrence (7 m)	Lung metastasis (10 m) Dead (22 m)	Lymph node metastasis (14 m)	R.F.

EBV, Epstein-Barr virus; EBER-ISH, EBV-encoded small RNA-*in situ* hybridization; EBNA2, EBV nuclear antigen 2; HPV, human papilloma virus; IHC, immunohistochemistry; LMP1, latent membrane protein 1; R.F., recurrence-free

Table 2. Literature review of p16-negative cervical squamous cell carcinoma (SCC) with HPV analyses

Author	Year	Total number of cases	Histology (number of cases)	p16 antibody clone	HPV (number of cases)	TS-PCR	Consensus PCR
Agoff [10]	2003	4	SCC (4)	E6H4	Negative	Yes	Multiple
Volgareva [11]	2004	1	SCC (1)	E6H4	HPV16 (1)	Yes	No
Tsai [12]	2013	1	Mimicking giant immature condyloma (1)	Not available	Negative	No	Single
Rodriguez [13]	2015	2	Keratinizing (1) Non-keratinizing (1)	E6H4	Negative	Yes	Single
Nicolas [14]	2019	5	Keratinizing (1) Non-keratinizing (3) Sarcomatoid (1)	E6H4	Negative	Yes	No
Liu [15]	2019	3	Papillary SIL + invasion (3)	E6H4	HPV6 (3)	Yes	Multiple
Our case	2019	5	Non-keratinizing (2) Non-keratinizing, condylomatous (1) Keratinizing (2)	E6H4	HPV56 (1) HPV6 (1) Unknown genotype (1)	Yes	Multiple

HPV, human papilloma virus; SIL, squamous intraepithelial lesion; TS-PCR, type-specific polymerase chain reaction;