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Etiological role of human papillomavirus infection in the development of penile cancer

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ABSTRACT

Objective: To examine the association between human papillomavirus (HPV) infection and penile cancer among Japanese patients.
Methods: Thirty-four patients with penile cancer were enrolled in this study. DNA was extracted from paraffin-embedded tumor tissue samples, and HPV-DNA tests and genotyping were performed. For all of the samples, in situ hybridization (ISH) was performed to locate HPV-DNA in tumor tissue. Furthermore, expression levels of p16-INK4a, mini-chromosome maintenance protein 7 (mcm-7), HPV-L1, and Ki-67 were analyzed using immunohistochemical methods.
Results: HPV and high-risk (HR)-HPV were detected in 14 (41.1%; 95% confidence interval (CI) 24.6–57.7%) and 12 (35.2%; 95% CI 19.2–51.4%) cases, respectively. HPV16 was the most frequently detected HPV type. Among the HR-HPV-positive cases, a punctate HR-HPV-DNA signal pattern was detected by ISH in tumor cell nuclei. P16-INK4a was expressed in 66.7% (95% CI 42.8–90.1%) of HR-HPV-positive cases and was significantly more frequent and stronger in HR-HPV-positive cases than in HPV-negative cases. There was no significant difference in the occurrence or distribution of mcm-7 or Ki-67 expression between HPV-positive and HPV-negative cases. HPV-L1 expression was not observed in any of the cases examined.
Conclusions: HPV infection may have had an etiological role in 41% of the examined cases of penile cancer in Japan.

Introduction

Human papillomavirus (HPV) is a common sexually transmitted infection (STI) (Smith et al., 2008) and a well-known cause of cervical cancer (Paavonen, 2007). Several recent studies have demonstrated that HPV infection may be involved in the development of malignant tumors other than cervical cancer, including oral, pharyngeal, anal, and skin cancers (Ang et al., 2010; Accardi et al., 2014; Barzał-Nowosielska et al., 2001; Hoots et al., 2009). In fact, it has been estimated that approximately 10% of cancer cases worldwide are associated with HPV infection (zur Hausen, 1996). Among urogenital malignancies, penile cancer is most likely to be associated with HPV infection, as confirmed by many epidemiological studies (Alemany et al., 2016; Backes et al., 2009; Miralles-Guri et al., 2009).

The incidence of penile squamous cell carcinoma, a rare malignancy of the urogenital system, shows geographic variations (Parkin and Bray, 2006; Calmon et al., 2011). It represents a significant public health hazard in developing countries such as Brazil, Uganda, and Puerto Rico. Phimosis, HPV infection, smoking, HIV infection, and STIs may cause penile cancer (Calmon et al., 2011; Larke et al., 2011). It is currently widely accepted that HPV infection is present in approximately half of all patients with penile cancer.

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Transitional HPV infections can often be found at genital sites in episomal forms, which are present as simple infections with many viral loads (Doorbar, 2006). On the other hand, persistent HPV infection often results in integration of the viral genome into the host genome, which can promote the development of cancer (Klaes et al., 2001; Shai et al., 2007). Therefore, verification of HPV-DNA integration into the host genome is essential to demonstrate an etiological role of HPV infection in penile carcinogenesis. Hence, the objectives of this study were to examine HPV infection status by detecting HPV-DNA locations in tumor tissue and to investigate the expression of certain proteins associated with the oncogenic process, in order to determine a possible role of HPV infection in the development of penile cancer.

Patients and methods

Subjects

Thirty-four patients who had undergone a partial or total penectomy for penile cancer at Kanazawa University Hospital or associated facilities were enrolled in this study. The diagnosis of penile cancer had been made by an experienced pathologist at each institution. Formalin-fixed, paraffin-embedded tumor tissue samples of all participants were collected. Written informed consent for the use of these samples was obtained from all participants, in accordance with a protocol approved by the Ethics Committee of Kanazawa University Graduate School of Medical Science.

HPV-DNA testing and genotyping

Penile tumor tissue in the slides was initially identified by hematoxylin–eosin staining. Next, DNA was extracted from each paraffin-embedded tumor tissue sample by micro-dissection using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA, USA). The DNA quality of all samples was confirmed by amplification of the β-globin gene by PCR analysis.

HPV-DNA testing and genotyping were performed using an HPV GenoArray kit (HybriBio; HybriBio Ltd, Hong Kong) according to the manufacturer's protocol. This assay can be optimized to detect 37 different HPV types, including 15 high-risk (HR) HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68), six low-risk (LR) HPV types (6, 11, 42, 43, 44, and CP8304), and 16 probably LR types (26, 34, 40, 54, 55, 57, 61, 67, 69, 70, 71, 72, 73, 82, 83, 84) by flow-through hybridization technique. After specific DNA was amplified by PCR, the amplified DNA samples were heat-denatured and then hybridized with specific HPV probes located on the membrane. The HPV type was determined by visualization of blue spots at the location of each HPV-type probe on the membrane using enzymatic immunoassay methods (Shigehara et al., 2010). The average detection limit of HPV-DNA in this array is around 300 copies/μl of the specific HPV-DNA target. This array has shown good concordance with results obtained by the Qiagen Hybrid Capture II procedure and the Amplicor HPV tests for HPV-type detection.

In situ hybridization (ISH) of HPV-DNA

For all samples, ISH was performed to detect HPV-DNA locations in the tumor tissue using a commercial HPV detection kit (Dako GenoPoint System K0620; Dako, Carpinteria, CA, USA) (Shigehara et al., 2011). A wide-spectrum probe (Y1404; Dako) for 13 HR-HPV-DNA (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) was hybridized with denatured DNA on the tissue slides for 24 h. HPV-DNA signals were visualized as brownish diaminobenzidine (DAB) staining, and hematoxylin was used to counterstain the cell nuclei in all specimens.

ISH signals were evaluated according to the following criteria: 'negative' (no signal; −), 'mild' (focal HPV-DNA signals in nuclei of tumor cells; 1+), 'moderate' (focal to diffuse signals; 2+), and 'strong' (diffuse signals; 3+).

Immunohistochemistry (IHC) for HPV-related proteins

The IHC analysis was performed using a Dako ChemMate Envision horseradish peroxidase/DAB universal kit (K5007) according to the manufacturer's protocol (Shigehara et al., 2011).

After deparaffinization of the samples, antigen retrieval was performed by heating tissue sections for 40 min at 95 °C, pH 9.0, in retrieval solution (S2367; Dako), after which the samples were cooled at room temperature for 20 min. After blocking endogenous peroxidase activity by addition of 3% hydrogen peroxide for 5 min, the sections were incubated with primary antibodies at room temperature for 30 min. The following primary antibodies were used: mouse monoclonal antibodies against p16-INK4a (Immuno-Biological Laboratories Co., Ltd, Gunma, Japan), against mini-chromosome maintenance protein 7 (mcm-7; Abnova, Taipei, Taiwan), against Ki-67 (Dako, Glostrup, Denmark), and against HPV-L1 protein (Viroactiv; Virofem Diagnostica GmbH, Mainz, Germany); they were applied at dilutions of 1:100, 1:100, 1:100, and 1:500, respectively. Next, the specimens were incubated for 30 min with peroxidase-labeled secondary IgG antibodies with a mixture of rabbit and mouse antibodies, as well as dextran polymers. Dark-brownish staining was obtained by peroxidase and DAB reactions, and sections were counterstained with hematoxylin.

For each protein, the expression levels were scored on the following semiquantitative scale: 'negative' (<5% of cells were stained; −), 'weak' (5–25% of cells were stained; 1+), 'intermediate' (25–50% of cells were stained; 2+), and 'strong' (>50% of the cells were stained; 3+) (Wang et al., 2016). Each protein was considered 'positive' if IHC staining was observed in >5% of the tumor cells (expression level ≥1+). In addition, p16 upregulation was defined as a pattern of >25% stained cells (expression level ≥2+) (Alemany et al., 2016).

Statistical analysis

The expression of each protein by IHC analysis was compared using univariate logistic regression analysis, and the odds ratio (OR) and 95% confidence interval (CI) were calculated. Comparisons in the distribution scores of IHC between HPV-positive and HPV-negative cases were performed by Mann–Whitney *U*-test. All statistical analyses were performed using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA), and *p*-values of <0.05 were considered statistically significant.

Results

PCR analysis

Among the 34 patients with penile cancer, the median age was 62 years (range 22–94 years). Histopathological examination demonstrated squamous cell carcinoma in all cases. HPV-DNA and HR-HPV-DNA were detected in 14 (41.1%; 95% CI 24.6–57.7%) and 12 (35.2%; 95% CI 19.2–51.4%) cases, respectively (Table 1). HPV16 was most common (detected in nine cases; 64.3%), followed by HPV26 (two cases; 14.3%), HPV71 (two cases; 14.3%), and HPV33, 34, 35, and 68 (one case each; 7.1%). HR-HPV types were detected in 12 (85.7%) cases, whereas LR-HPV types were detected in two (14.3%). The two cases in which only the LR type was detected had multiple HPV-type infections. In contrast, single HPV-type infections were observed in all of the 12 cases with HR-HPV infection.

Table 1
Summary of HPV typing, ISH, and IHC in all subjects.

Number	Year	HPV type	ISH (patterns)	IHC			
				P16-INK4A	mcm-7	HPV-L1	Ki-67
1	2010	ND	–	–	3+	–	–
2	2010	ND	–	–	2+	–	1+
3	2008	16	3+ (punctate/diffuse)	3+	3+	–	1+
4	2007	ND	1+ (punctate)	1+	3+	–	–
5	2007	16	3+ (punctate/diffuse)	3+	3+	–	–
6	2005	ND	–	–	–	–	–
7	2003	16	3+ (punctate)	–	1+	–	–
8	2002	ND	–	3+	3+	–	–
9	2000	ND	–	–	1+	–	–
10	1999	ND	–	–	–	–	–
11	2009	33	2+ (punctate)	2+	2+	–	–
12	2008	68	3+ (punctate)	1+	–	–	–
13	2009	ND	–	–	–	–	–
14	2006	ND	–	–	–	–	–
15	2006	ND	–	–	2+	–	–
16	2010	16	1+ (punctate)	–	–	–	–
17	2010	16	3+ (punctate)	1+	1+	–	–
18	2016	16	1+ (punctate/diffuse)	–	1+	–	2+
19	2016	ND	–	–	1+	–	1+
20	2017	ND	–	–	1+	–	3+
21	2014	16	1+ (punctate)	3+	3+	–	1+
22	2013	16	1+ (punctate)	2+	2+	–	3+
23	2011	16	1+ (punctate)	2+	2+	–	–
24	2011	ND	–	–	1+	–	–
25	2009	ND	–	–	3+	–	2+
26	2008	ND	–	–	1+	–	1+
27	2008	ND	–	1+	1+	–	–
28	2016	ND	–	–	2+	–	–
29	2013	ND	–	–	3+	–	3+
30	2015	26, 71	–	–	3+	–	2+
31	2016	26, 34, 71	2+ (punctate)	2+	2+	–	3+
32	2016	35	2+ (punctate)	–	2+	–	3+
33	2016	ND	–	–	1+	–	3+
34	2017	ND	–	–	2+	–	1+

HPV, human papillomavirus; ISH, in situ hybridization; IHC, immunohistochemistry; ND, not detected.

Observation of HPV-DNA signals based on ISH analysis

ISH analysis demonstrated that HR-HPV-DNA signals could be detected in tumor cell nuclei from all of the 12 cases in which HR-HPV was detected by PCR analysis (Figure 1A); strong signals occurred in five cases, moderate signals in two, and mild signals in five. The ISH-based HPV-DNA signals revealed a punctate pattern (Figure 1B). Notably, HR-HPV-DNA signals were also positive in two other cases: one with LR-HPV infection (case 31; moderate signal) and another HPV-negative case (case 4; mild signal) (Table 1).

IHC analysis

P16-INK4a was expressed in eight of 12 cases (66.7%; 95%CI, 42.8–90.1%) with HR-HPV-DNA (Figure 2A), and P16-INK4a upregulation was detected in six of the HR-HPV-positive cases and two of the HR-HPV-negative cases (Table 2). Conversely, 18 out of 22 cases (81.8%; 95% CI 65.6–98.0%) without p16-INK4a expression did not show an HR-HPV infection. On the other hand, mcm-7 expression was observed in 28 cases (Figure 2B), which included 10 HR-HPV-positive cases and 18 HR-HPV-negative cases. Expression of HPV-L1, a capsid protein of HPV particles, was not observed in any of the cases, regardless of HPV

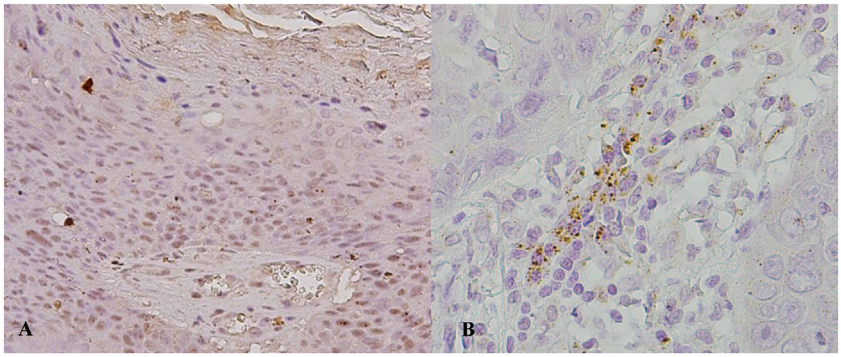

Figure 1. (A) In situ hybridization (ISH) findings of HPV-positive penile cancer (case 3; HPV16) shown at 200× magnification. Moderately intense HPV-DNA signals were observed in tumor-cell nuclei. (B) Punctate ISH signals, shown at 400× magnification.

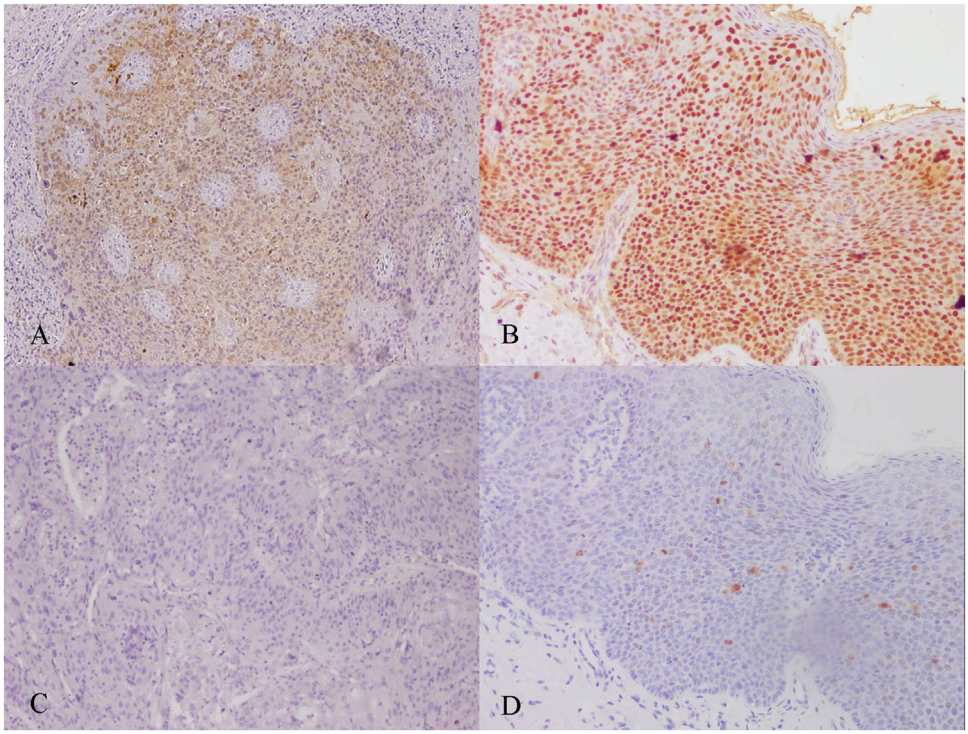

Figure 2. Histopathological findings of (A) p16-INK4a, (B) mcm-7, (C) HPV-L1, and (D) and Ki-67 expression in HPV16-positive penile cancer samples (case 3: HPV16-positive, 200× magnification). P16-INK4a and mcm-7 were expressed widely in the nuclei and cytoplasm of tumor cells, whereas Ki-67 was only expressed in nuclei. HPV-L1 was not expressed in the tumor tissue at all.

infection status (Figure 2C). Ki-67, which is an excellent marker of cell growth, was expressed in five (41.7%; 95% CI 17.2–66.1%) of the 12 HR-HPV-positive cases and in 10 (45.5%; 95% CI 26.2–64.7%) of the 22 LR-HPV-positive or HPV-negative cases (Figure 2D).

P16-INK4a expression occurred significantly more frequently in HR-HPV-positive cases than in HPV-negative ones (OR 9.0, 95% CI 1.8–45.3; $p = 0.01$), and P16-INK4a upregulation was also more frequently observed in HR-HPV-positive cases (OR 10.0, 95% CI 1.6–63.1; p = 0.01) (Table 2). In addition, comparison of distribution scores between HR-HPV-positive and HR-HPV-negative cases, revealed that p16-INK4a expression was significantly stronger in HPV-positive cases ($p = 0.02$) (Figure 3). On the other hand, there was no significant between-group difference in frequency or distribution of mcm-7 or Ki-67 expression (Table 2, Figure 3).

Discussion

In this study including Japanese patients with penile cancer, HPV was detected in 41.1% of cases; moreover, HR-HPV-DNA signals could be observed based on ISH in all of the cases in which HPV was detected by PCR analysis. A wide range of HPV prevalence rates has been found in penile cancer in some previous studies in Japan, including those by Yanagawa et al. (12%; 3/76 cases), Suzuki et al. (54%; 7/13 cases), and Senba et al. (76.5%; 5/39 cases), by using PCR or ISH analysis (Yanagawa et al., 2008; Suzuki et al., 1994; Senba et al., 2010). A systematic review also demonstrated a large variation of HPV prevalence in penile cancer, with reported detection rates in 30 studies of between 19.7% and 100% (Backes et al., 2009). Another review article including 1466 penile cancer samples found an overall HPV prevalence of 46.9% (Miralles-Guri et al., 2009). Recently, a retrospective, large-scale study from 2016 of 1010 penile cancer samples from 25 countries demonstrated that HPV could be detected by PCR in 33.1% of cases (Alemany et al., 2016). Furthermore, a very recent meta-analysis, which was conducted to examine the association between HPV DNA and survival in men with penile cancer, found that the overall prevalence of HPV was 39.4% in 1107 patients (Sand et al., 2018). The wide range of reported HPV prevalence rates is likely to

Table 2
Comparison of expression of various proteins based on HR-HPV status.

IHC analysis		HR-HPV status		OR (95% CI)	*p*-Value
		Positive ($n = 12$)	Negative ($n = 22$)		
p16-INK4a	Upregulation	6	2	10.0 (1.6–63.1)	0.01
	Positive	8	4	9.0 (1.8–45.3)	0.01
	Negative	4	18		
mcm-7	Positive	10	18	1.1 (0.2–7.2)	1.00
	Negative	2	4		
HPV-L1	Positive	0	0	NA	NA
	Negative	12	22		
Ki-67	Positive	5	10	0.9 (0.2–3.6)	0.88
	Negative	7	12		

HR, high-risk; HPV, human papillomavirus; IHC, immunohistochemistry; OR, odds ratio; CI, confidence interval; NA, not analyzed.

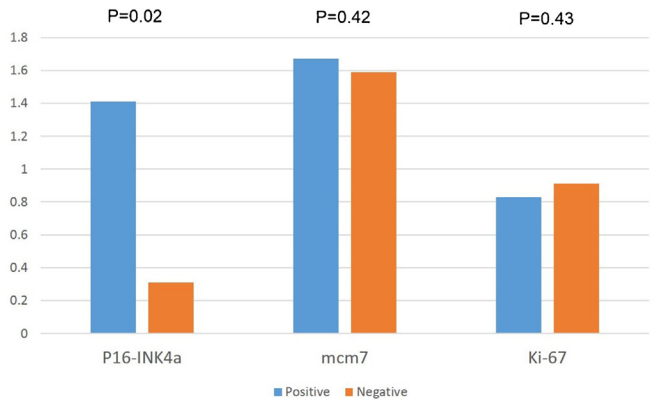

Figure 3. Comparison of the distribution scores between HR-HPV-positive cases and HR-HPV-negative cases. P16-INK4a was expressed more widely in HPV-positive cases. On the other hand, there were no significant between-group differences in frequency or distribution of mcm-7 or Ki-67 expression.

be greatly dependent on the geographical location, sample type examined, sample size, and methods of detection.

In the present study, it was found that HPV16 was the most frequent HPV type in penile cancer, whereas HPV types 33, 35, and 68 were each detected in only one case. Indeed, in many studies, HPV16 has been the most commonly detected type in penile cancer samples. A systematic review of relevant studies reported that HPV16 was the most frequently detected type (30.8%), followed by HPV6 (14.0%) and HPV18 (13.8%) (Backes et al., 2009). A large study reported that HPV16 was the most frequently detected type in penile samples, followed by HPV types 6, 35, 45, and 33 (Alemany et al., 2016). Likewise, HPV16 was most frequently detected in HPV infection-associated neoplasms, such as cervical, oropharyngeal, and anal cancer (Hoots et al., 2009; Ang et al., 2010). HPV16 is most likely to be associated with the development of cancer, regardless of the origins of cancer. Indeed, it has been well documented that HPV16 infection is most likely to be persistent among men with genital HPV infections (Giuliano et al., 2011). In addition, a previous study by the present authors' group demonstrated that subclinical HPV infection-associated cytological changes, e.g., koilocytosis, multinucleation, hyperchromatism, and mild atypical cells, could be found in men with penile HR-HPV infections (Shigehara et al., 2014). The persistence of genital HR-HPV infections may result in the development of penile cancer.

HPV infection essentially affects the squamous epithelium, effectively in two ways: either as simple viral infection or as viral-associated cancerous infection (Klaes et al., 2001; Shai et al., 2007; Doorbar, 2006). Simple viral infection (episomal infection), which represents largely transient HPV infections in the squamous epithelium, is responsible for lesions such as condyloma and mild dysplasia. On the other hand, in viral-associated cancerous infection, the viral genome integrates into the host genome with the expression of some viral oncogenes for malignant transformation. Generally, the most common anatomical site of HPV infection in men is the penile glans, and simple viral infection is often observed in the glans (Giuliano et al., 2011; Shigehara et al., 2010). Therefore, it is important to demonstrate evidence of HPV-DNA integration into tumor tissue to confirm a role of HPV infection in penile carcinogenesis.

In the present ISH analysis, HR-HPV-DNA signals could be observed in tumor cell nuclei in all HR-HPV-positive cases. Interestingly, a predominantly punctate signal pattern could be observed in most of the HPV-positive cases, whereas diffuse signal patterns were rarely seen. In cervical cancer, diffuse signal patterns generally represent an episomal state of the HPV genome in host cells, while punctate patterns indicate HPV genome integration into the host cells (De Marchi Triglia et al., 2009; Cooper et al., 1991). In particular, punctate signals are often observed in high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer, whereas diffuse signals are present in low-grade CIN and condyloma acuminatum. The results of the ISH analysis in the present study suggest that HPV-DNA may have been integrated into the tumor cells of penile cancer, which suggests an association between HPV infection and carcinogenesis. However, signal patterns are evaluated subjectively, and the accuracy of signal pattern detection has not been adequately validated for penile cancer. Therefore, further verification may be required, for instance by including analyses of more varied types of penile cancer, such as penile intraepithelial neoplasia (PIN) and condyloma, as well as of normal tissues.

P16-INK4a is a tumor-suppressor gene that suppresses the inactivation of Rb (retinoblast) protein and whose expression increases as the cell cycle progresses. mcm-7, a component of a cellular DNA-helicase complex, is coded by a gene that is responsive to transcription factor E2F, which promotes cell division by inactivation of Rb protein, similarly to the actions of p16-INK4a. The HPV-E7 protein, which is an oncogenic protein of HPV, can bind and inactivate Rb protein and cause carcinogenesis with promotion of cell-cycle progression. Hence, cervical cancer associated with HPV infections shows increased cell proliferation with p16-INK4a and mcm-7 overexpression; these proteins are therefore used as surrogate markers for HPV-E7 expression of HR-HPV in cervical and oropharyngeal cancers (Brake et al., 2003; Klaes et al., 2001; Shai et al., 2007; Zhang et al., 2013). Consequently, we also evaluated p16-INK4a and mcm-7 expression levels by IHC analysis to determine a potential role of HPV infection in carcinogenesis.

It was found that p16-INK4a was frequently expressed in cases with HR-HPV-DNA, and p16-INK4a upregulation was also frequently detected in HR-HPV-positive cases. In addition, a significantly higher expression distribution score was observed in cases with HR-HPV-DNA than in cases without. Some previous studies have suggested that p16-INK4a could serve as a surrogate marker for penile cancer associated with HR-HPV infections. A previous study of 58 subjects demonstrated that the sensitivity and specificity of p16-INK4a expression as a predictor of HR-HPV infection was 100% and 57%, respectively (Steinestel et al., 2015). Another large multicenter study including 1010 samples showed that p16-INK4a was expressed in 80% of cases with HR-HPV positivity (Alemany et al., 2016). The present findings of p16-INK4a expression in cases with HR-HPV infections may be evidence to support a potential role of HPV infection in penile carcinogenesis, in line with the punctate pattern of staining obtained by ISH analysis. However, p16-INK4a expression was also observed in HPV-negative cases. Actually, it was found that p16-INK4a was expressed in four cases without detectable HR-HPV. Indeed, it was also reported in a previous study that p16-INK4a was expressed in about 15% of HPV-negative cases (Alemany et al., 2016). Therefore, its specificity to predict HPV-DNA positivity in penile cancer may not always be sufficient, making it essential to combine PCR and IHC analyses of penile cancer samples.

On the other hand, mcm-7 expression was observed in many cases regardless of HPV-DNA positivity, and no significant differences in distribution scores were found between the HPV-positive and HPV-negative groups. mcm-7 expression was not associated with HPV infection in penile cancer. The role of mcm-7 expression in penile cancer has not yet been elucidated. mcm-7 might be expressed by another pathway unrelated to HPV infection.

HPV-L1 is a viral capsid protein that is expressed during the final stage of HPV particle formation. Thus, HPV-L1 is known as a marker of viral particle formation (Doorbar, 2006). In general, the L1 protein is not expressed during the carcinogenetic stage with

increased expression of the oncogenic genes E6 and E7 of HPV; in contrast, L1 expression is often observed during non-integrated HPV infections (episomal infections), such as simple transitional HPV infections or low-grade CIN. In the present study, no increases in HPV-L1 expression were observed in HPV-positive cases. This may suggest an etiological role of integrated HPV infections in the development of penile cancer, which is consistent with the findings from both the ISH analysis and p16-INK4a expression.

Ki-67, which is commonly used as a marker of proliferation, is associated with tumor grade and lymph node metastasis in penile cancer (Berdjis et al., 2005; May et al., 2013; Protzel et al., 2007). Alternatively, this protein may serve as a surrogate marker for cell-cycle deregulation by transforming HPV infections in the cervical epithelium, and might be an important triage tag for women with HR-HPV infection (Polman et al., 2017). We observed Ki-67 expression in only 41.6% of HPV-positive cases, and no significant difference in Ki-67 expression was observed between HR-HPV-positive cases and HR-HPV-negative cases. Furthermore, no significant difference in Ki-67 distribution scores was found. The present results suggest that Ki-67 expression may not be a suitable surrogate marker of HPV infection in penile cancer. In contrast to the results from our study, only two previous studies have demonstrated that Ki-67 might be a potential surrogate marker for HPV infection (Protzel et al., 2007; Stankiewicz et al., 2012). However, only a few studies have explored the relationship between Ki-67 and HPV infection in penile cancer, and these have included a relatively small number of subjects. Therefore, further studies with larger numbers of subjects are needed to reach definite conclusions.

It should be noted that the present study had a number of limitations. First, a limited number of subjects were included. However, the study was enhanced by including various molecular analyses, such as PCR, ISH, and IHC. Indeed, viral integrated infection could be suggested in HPV-positive cases based on results obtained by ISH and IHC, such as p16-INK4a and HPV-L1, and this may suggest an etiological role of integrated HPV infections in the development of penile cancer. Second, there are no standards for the cut-off levels and semiquantitative categorizations of the expression levels of each protein. In particular, the cut-off levels of p16-INK4a expression/upregulation have varied widely in different studies, ranging from 5% to 50% (Klaes et al., 2001; Shigehara et al., 2011; Alemany et al., 2016; Wang et al., 2016). On the other hand, in some cases the results from the different molecular analyses did not completely agree with each other. Therefore, it remains necessary to verify whether p16-INK4a could serve as a surrogate marker of HPV-E7 expression, as in the similar case of HPV-positive cervical cancer. Indeed, increased mcm-7 and Ki-67 expression was not observed in cases with HPV-positive penile cancer, which obviously differs from cervical cancer. In addition, clinical data regarding cancer progression and prognosis were not available in our study. Therefore, further studies with larger numbers of subjects and a more complete panel of clinical data are required to confirm an etiological role of HPV infection in the development of penile cancer.

In conclusion, this study showed that HPV-DNA could be detected in 41% of the Japanese patients with penile cancer examined. Moreover, results obtained by molecular methods (i.e., ISH and IHC) suggest that HPV-DNA plays an etiological role in the development of penile cancer.

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Ethical approval

The study protocol was approved by the Ethics Committee of Kanazawa University Graduate School of Medical Science.

Conflict of interest

No conflict of interest.

Author contributions

Jiro Sakamoto, Kazuyoshi Shigehara, Kazufumi Nakashima, and Shohei Kawaguchi contributed to the study design and organized the multicenter study. Jiro Sakamoto and Kazuyoshi Shigehara were primarily responsible for writing the manuscript and data analysis. Takao Nakashima, Masayoshi Shimamura, Taku Kato, Mitsuru Yasuda, Toru Hasegawa, Yoshitomo Kobori, Hiroshi Okada, and Takashi Deguchi collected clinical samples and data. Kouji Izumi, Yoshifumi Kadono, and Atsushi Mizokami contributed to editing of the manuscript.

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