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The etiologic role of human papillomavirus in penile cancers: a study in Vietnam

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Background: We investigated the aetiologic role of human papillomavirus (HPV) in 120 penile squamous cell carcinomas (PSCCs) from Vietnam.

Methods: Human papillomavirus DNA was detected by PCR using SPF10 primers and a primer set targeting HPV-16 E6. The INNO-LiPA HPV genotyping kit was used to determine genotype. Human papillomavirus-16 viral load and physical status were determined by real-time PCR. P16^{INK4A} protein expression was investigated by immunohistochemistry.

Results: Human papillomavirus DNA was detected in 27 of 120 (23%) PSCCs. The most frequently detected genotype was HPV-16 (24 of 27 cases, 89%). In 16 of 18 (89%) HPV-16-positive cases, the HPV DNA was considered to be integrated into the host genome. The geometric mean of the HPV-16 viral load was 0.4 copies per cell. P16^{INK4A} overexpression was significantly related to PSCCs infected with high-risk HPV ($P=0.018$) and HPV-16 copy numbers ($P<0.001$).

Conclusion: Human papillomavirus-16 DNA integration and p16^{INK4A} overexpression in high-risk HPV detected PSCCs suggested an aetiologic role of high-risk HPV in the development of PSCCs.

Penile cancer development is a multi-factorial process involving poor genital hygiene, phimosis, human papillomavirus (HPV), chronic inflammatory and premalignant conditions, and smoking (Misra *et al*, 2004). According to a systematic review, human papillomavirus (HPV) DNA was detected in 48%, on average, of penile squamous cell carcinomas (PSCCs) and the most frequent HPV-related histotype (66%) was basaloid PSCC (Backes *et al*, 2009).

The integration of high-risk HPV genome into the host genome is suspected to be an important event for malignant transformation and cancer progression (Wentzensen *et al*, 2004; Williams *et al*, 2011). It usually disrupts the E2 gene, a suppressor of the E6/E7 promoter, leading to the overexpression of viral oncogenes E6 and E7. The high viral load, frequently observed in cervical cancer, is also a determinant of cancer development (Wu *et al*, 2006).

The overexpression of p16^{INK4A} is a useful biomarker for evaluating the aetiologic role of HPV because HPV-E7 disturbs the p16^{INK4A}/cyclin D/Rb pathway, leading to the accumulation of p16^{INK4A} (Narisawa-Saito and Kiyono, 2007). In PSCC, however, the association between p16^{INK4A} overexpression and HPV presence is still unestablished (Ferreux *et al*, 2003; Poetsch *et al*, 2011; Stankiewicz *et al*, 2011a).

To understand the aetiologic role of HPV in the development of PSCCs, we examined the presence, genotype, viral load and physical status of high-risk HPV, and p16^{INK4A} expression in PSCCs in Vietnam.

MATERIALS AND METHODS

Study subjects. One hundred twenty paraffin-embedded PSCC specimens diagnosed at the National Cancer Hospital (Hanoi, Vietnam) between 2005 and 2010 were examined. Seventeen

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cervical cancer specimens obtained at the same hospital during the same period were used as positive controls as HPV is a necessary cause of cervical cancers (zur Hausen, 2002). Histological subtypes were confirmed according to the World Health Organization histological classification of PSCCs (Cubilla *et al*, 2004). On the basis of TNM classification (Pizzocaro *et al*, 2010), clinical stage was divided into four stages by one of the authors (HD). This study was approved by the Institutional Review Board of Kagoshima University Graduate School of Medical and Dental Sciences.

Human papillomavirus detection and genotyping. Extracted DNAs from tissue specimens were checked for their qualities and the absence of PCR inhibitors by PCR for β-globin (Khan *et al*, 2008). Human papillomavirus DNA was detected by PCR using SPF10-biotinylated primers and a HPV-16-E6-specific primer set (Khan *et al*, 2008). Human papillomavirus typing was performed using the INNO-LiPA HPV Genotyping Extra test (Innogenetics, Ghent, Belgium) (Kleter *et al*, 1999), which can identify 28 genotypes: HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, -82, -26, -53, -66, -6, -11, -40, -43, -44, -54, -70, -69, -71 and -74.

Quantitative real-time PCR. To examine the viral load and the physical status of HPV-16, all HPV-16-positive samples were subjected to quantitative real-time PCR with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and 2x QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). Details of the procedure were reported in the previous

Table 1. Frequencies of HPV-positive cases by clinicopathological characteristics

Clinicopathological characteristics	All *N* (%)	HPV-negative *N* (%)	HPV-positive *N* (%)	*P*-value[a]
Number of cases	120 (100)	93 (77)	27 (23)	
Mean age at diagnosis (95% CI)	53 (51, 57)	54 (51, 57)	51 (47, 55)	0.277[b]
Age at diagnosis (years)				
⩽45	32	24 (75)	8 (25)	0.273
46–55	47	35 (74)	12 (26)	
56–65	17	12 (71)	5 (29)	
66 and above	24	22 (92)	2 (8)	
Histological subtype				**0.354**
Basaloid	3	1 (33)	2 (67)	
Keratinising (usual type)[c]	83	64 (77)	19 (23)	
Non-keratinising	20	17 (85)	3 (15)	
Verrucous	8	6 (75)	2 (25)	
Warty(condylomatous)	2	2 (100)	0 (0)	
Undetermined[d]	4	3 (75)	1 (25)	
Histological grade				**0.104**
1	61	52 (85)	9 (15)	*P* for trend = 0.120[e]
2	36	25 (70)	11 (30)	
3	19	13 (68)	6 (32)	
Undetermined[d]	4	3 (75)	1 (25)	
Stage				**0.550**
1	1	1 (100)	0 (0)	*P* for trend = 0.815[e]
2	29	22 (76)	7 (24)	
3	23	16 (70)	7 (30)	
4	6	6 (100)	0 (0)	
Unknown	61	48 (79)	13 (21)	
Phimosis history				**0.433**
No	11	10 (91)	1 (9)	
Yes	54	40 (74)	14 (26)	
Unknown	55	43 (78)	12 (22)	
Anatomic localisation				**0.180**
Penile glans	59	46 (78)	13 (22)	
Penile preputium	3	1 (33)	2 (67)	
Both glans and preputium	4	3 (75)	1 (25)	
Unspecified/unknown	54	43 (80)	11 (20)	
Smoking history				**1.00**
No	44	35 (80)	9 (20)	
Yes	12	10 (83)	2 (17)	
Unknown	64	48 (75)	16 (25)	

Abbreviations: CI = confidence interval; HPV = human papillomavirus.
[a]*P*-values for heterogeneity, except unknown/undetermined cases, were obtained by Fisher's exact test.
[b]*P*-value was obtained by Student's *t*-test.
[c]One case with mixed keratinising-verrucous was included.
[d]Histological subtype was undetermined in four cases due to tissue shortage.
[e]*P* for trend was obtained by Cochran–Armitage trend test.

study (Khan *et al*, 2008). The physical status of the HPV-16 was determined by the HPV-16 E2/E6 ratio (Peitsaro *et al*, 2002). A lack of E2 amplification (E2/E6 ratio = 0) represents HPV-16 DNA integration into the host genome. When the E2/E6 ratio was equal to or higher than unity (E2/E6 ratio $\geqslant 1$), the HPV-16 genome was considered as an episomal form, and the rest ($0 < \text{E2/E6 ratio} < 1$) was considered as a mixed form (mixture of episomal and integrated forms).

Immunohistochemistry for p16^{INK4A}. The immunohistochemistry was conducted, using the mouse monoclonal antibody against p16^{INK4A} (1 : 150 dilutions, 551153, BD Pharmingen, Tokyo, Japan). Details of the procedure were reported in the previous study (Baba *et al*, 2010). The p16^{INK4A} expression was classified into the following four groups: <10%, 10–49%, 50–89% and $\geqslant$90%. The cases with $\geqslant$10% carcinoma cells stained positively were classified as positive.

RESULTS

The β-globin was detected in all samples, indicating that DNA was available for molecular analysis. Twenty-seven of 120 (23%) PSCCs, including two of three (67%) basaloid PSCCs, were HPV positive (Table 1). Twenty-three HPV-positive cases were detected by SPF10 primers, and additional four cases by a HPV-16-specific primer set (PC-1, PC-4, PC-7 and PC-8 in Supplementary Table 1). The HPV prevalence did not differ by any clinicopathological parameters. In cervical carcinomas, HPV DNA was detected in 94% (16 out of 17) cases.

Human papillomavirus-16 was detected in 24 of 27 PSCCs. Other HPV genotypes detected included HPV-18, -11, -33 and -58 in one case each. Two cases had multiple infections: HPV-16/-58 and HPV-16/-18 (Supplementary Table 1).

Human papillomavirus-16 E6 DNA was quantified in 18 of 24 HPV-16-positive PSCCs (Supplementary Table 1). The geometric means of E6 copies per cell were 0.4 in PSCCs and 3.0 in cervical cancers. Human papillomavirus DNA was in the integrated form in seven (39%), the mixed form in nine (50%) and the episomal form in two (11%) cases. In 13 HPV-16-positive cervical cancers, HPV integrated, mixed and episomal forms were found in four (31%), three (23%) and six (46%) cases, respectively.

Eighteen of 25 high-risk HPV (16 HPV-16, one HPV-16/-58 and one HPV-33)-positive cases and 26 randomly selected HPV-negative PSCCs were subjected to immunohistochemistry. Seven high-risk HPV-positive cases (PC-4, PC-5, PC-11, PC-14, PC-16, PC-17 and PC-22) were not examined due to tissue shortage.

Although p16^{INK4A} had both nuclear and cytoplasmic immunoreactivity (Figure 1A and D), samples showing cytoplasmic immunoreactivity alone (Figure 1B and E) were not regarded as positive because the functionally activated p16^{INK4A} was translocated into the nucleus. P16^{INK4A} was expressed in 10 of 44 (23%) PSCCs (Supplementary Table 2). Human papillomavirus-positive PSCCs tended to show a frequent p16^{INK4A} nuclear expression (Supplementary Table 2, $P = 0.018$). Strong p16^{INK4A} nuclear expression was more frequently observed in HPV-16-positive PSCCs with a viral load $\geqslant 1$ than those with a viral load < 1 and HPV-negative PSCCs (Table 2, P for trend < 0.001). Although all basaloid and non-keratinising types had high viral loads, 83% of the keratinising type harboured low viral loads (Table 2, $P = 0.032$). P16^{INK4A} expression was not related to other factors, including tumour grade or stage (Supplementary Table 2).

DISCUSSION

In the present study, p16^{INK4A} overexpression was frequently observed in high-risk HPV-positive PSCCs, which is consistent with the findings in cervical cancers (Hwang and Shroyer 2012) and some studies of PSCCs (Ferreux *et al*, 2003; Stankiewicz *et al*, 2011a). Regarding HPV-negative PSCCs, p16^{INK4A} expression was frequently suppressed by p16^{INK4A} gene mutation or promoter hypermethylation (Poetsch *et al*, 2011). However, three HPV-negative PSCCs also showed p16^{INK4A} overexpression (Table 2), which might occur independently of HPV infection such as mutational inactivation of pRB (Marur *et al*, 2010). A high viral load of HPV-16 was also related to strong p16^{INK4A} nuclear

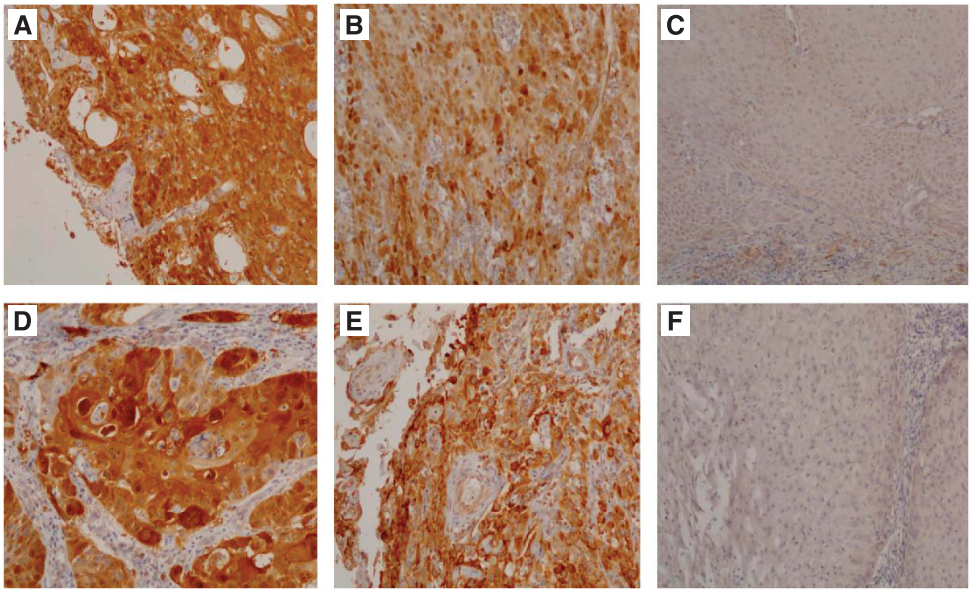

Figure 1. Representative examples of p16^{INK4A} immunostaining in PSCCs. (**A**) HPV positive, p16^{INK4A} both nuclear and cytoplasmic positive; (**B**) HPV positive, p16^{INK4A} cytoplasmic positive only; (**C**) HPV positive, p16 ^{INK4A} both nuclear and cytoplasmic negative; (**D**) HPV negative, p16 ^{INK4A} both nuclear and cytoplasmic positive; (**E**) HPV negative, p16^{INK4A} cytoplasmic positive only; (**F**) HPV negative, p16^{INK4A} both nuclear and cytoplasmic negative.

Table 2. Association of HPV-16 viral load with histological subtype and $p16^{INK4A}$ expression

		HPV-16 E6 viral load (per cell)		
Clinicopathological characteristics	**HPV negative**	**Low (<1) *N* (%)**	**High (⩾1) *N* (%)**	***P*-value[a]**
Histological subtype				**0.032**
Basaloid	1 (1)	0 (0)	2 (33)	
Keratinising (usual type)	64 (69)	10 (83)	2 (33)	
Non-keratinising	17 (18)	0 (0)	2 (33)	
Verrucous	6 (6)	1 (8)	0 (0)	
Warty(condylomatous)	2 (2)	0 (0)	0 (0)	
Undetermined	3 (3)	1 (8)	0 (0)	
Total	93 (100)	12 (100)	6 (100)	
$p16^{INK4A}$ expression level				**0.001**
<10%	23 (88)	8 (89)	0 (0)	*P* for trend <**0.001**[b]
10–49%	3 (12)	1 (11)	0 (0)	
50–89%	0 (0)	0 (0)	3 (100)	
⩾90%	0 (0)	0 (0)	0 (0)	
Total	26[c] (100)	9 (100)	3 (100)	

Abbreviation: HPV = human papillomavirus.
[a]*P*-values for heterogeneity, except unknown/undetermined cases, were obtained by Fisher's exact test.
[b]*P* for trend was obtained by linear-by-linear association test.
[c]26 HPV-negative samples were randomly selected from all HPV-negative samples.

expression and almost none of the cases with viral load <1 copy per cell showed $p16^{INK4A}$ overexpression. To our knowledge, this is the first study reporting this association. $P16^{INK4A}$ overexpression may predict HPV transcription activity as reported in tonsillar cancer (Hoffmann *et al*, 2010).

The median HPV-16 viral loads (ranges) were 60.1255 (10.7–1239) and 0.0355 (0.002–0.322) copies per cell in the high (⩾1 per cell) and low (<1 per cell) viral load groups, respectively. These ranges were similar to those in HPV-16 E6 mRNA-positive and -negative PSCCs, respectively (Heideman *et al*, 2007). Thus, approximately one copy per cell is a reasonable threshold to distinguish the viral transcription activity.

Relatively low viral loads in PSCCs might be due to the frequent HPV-16 integration because the viral load decreases after viral integration into the host genome (Berumen *et al*, 1995). The high frequency (89%) of HPV-16 integration in Vietnamese PSCCs (both integrated and mixed forms) is consistent with the findings by Tornesello *et al* (1997) and Kalantari *et al* (2008). Human papillomavirus integration, a marker of HPV-induced neoplasia, is not always found in cervical cancers (Cullen *et al*, 1991; Vernon *et al*, 1997). The discrepancy in HPV-16 integration rate between PSCCs and cervical cancers in the current study could be explained by the difference in tumour histological grades or aggressiveness, as HPV integration frequently occurs in a severe dysplastic lesion and invasive cervical carcinomas (Wentzensen *et al*, 2004). However, our sample size was too small to explore this hypothesis: only two PSCCs were in episomal form.

Demographic features and genetic backgrounds may contribute to the geographical difference of HPV prevalence in PSCCs worldwide. To date, there is no study reporting the HPV presence in PSCC in Vietnam. Although the HPV prevalence in Vietnamese PSCCs was relatively lower than the world average, a high frequency (94%) of HPV in cervical cancers indicated the appropriateness of our HPV DNA detection procedure.

Human papillomavirus presence was not significantly related to other PSCC risk factors including phimosis and smoking. Although clinical information was not obtained from nearly half of the study subjects, the HPV prevalence in these cases (20–25%) differed little from that of the entire group (23%). Thus, our negative finding was unlikely caused by biased information.

CONCLUSION

The aetiologic role of high-risk HPV in the development of PSCCs was suggested by its DNA integration into the PSCC genome and its association with $p16^{INK4A}$ overexpression. $P16^{INK4A}$ could be a biomarker for HPV-related PSCCs.

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