

RESEARCH ARTICLE

HPV Detection and Genotyping in Vulvar Squamous Cell Carcinoma in Northern Thailand

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Abstract

Background: The study was aimed to evaluate the prevalence and genotype distribution of HPV infection in vulvar squamous cell carcinoma (SCC) in northern Thailand and the clinicopathological difference with regard to HPV infection status. **Materials and Methods:** Formalin-fixed paraffin-embedded tissue samples of vulvar SCC diagnosed between January 2006 and December 2012 were collected. HPV infection was detected by nested polymerase chain reaction (PCR) with primers MY09/11 and GP5+/6+. HPV genotyping was performed using the Linear Array Genotyping Test, followed by type-specific PCR targeting the E6/E7 region of HPV16/18/52 if the Linear Array test was negative. The histologic slides of vulvar lesions and the medical records were reviewed. **Results:** There were 47 cases of vulvar SCC included in the study (mean patient age 57.9±13.2 years). HPV infection was detected in 29 cases (62%), all of which had single HPV infections. HPV16 accounted for 23 (49%). The patients with HPV-positive SCC had a significantly younger mean age than those with HPV-negative tumors (52.7 years vs 66.2 years, $p<0.001$). There was no significant difference in tumor stage distribution with regard to the status of HPV infection. The presence of vulvar intraepithelial neoplasia (VIN) of usual type (basaloid or warty) was significantly more frequent in HPV-positive cases compared with HPV-negative cases (62% vs 6%, $p<0.001$), whereas differentiated-type VIN was more common in HPV-negative cases (24% vs 0%, $p=0.019$). **Conclusions:** HPV infection was detected in 62% of vulvar SCC in northern Thailand. HPV16 was the predominant genotype similar to the data reported from other regions. HPV-positive SCC occurred in younger patients compared with HPV-negative SCC, and was associated with usual-type VIN. Vaccination against HPV16/18 may potentially prevent almost one half of vulvar SCC in northern Thailand.

Keywords: Vulva - squamous cell carcinoma - human papillomavirus (HPV) - genotyping - prevalence

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Introduction

Vulvar cancer is an uncommon type of malignant neoplasms in the female genital tract, accounting for 4-5% of all gynecologic cancers (Benedet et al., 2000). Squamous cell carcinoma (SCC) represents the most common histologic type that comprises almost 90% of vulvar cancers (Del Pino et al., 2013). Patients with early stage vulvar SCC have a favorable prognosis and can be successfully treated by surgery with a 10-year disease-specific survival of approximately 80-90% (Tabbaa et al., 2012).

Human papillomavirus (HPV) is known as an important cause of SCC of the uterine cervix. The role of HPV as an etiologic factor of SCC in the other sites of anogenital tract has been increasingly recognized and genotype distribution has been assessed in cervical lesions in Thailand (Natphopsuk et al., 2013; Swangvaree et

al., 2013). In vulvar carcinoma, the overall prevalence of HPV infection has been reported as 40.4%, which is lower than that of anal cancer in women (90.8%) and vaginal cancer (69.9%) (De Vuyst et al., 2009). HPV16 is the most common genotype in vulvar SCC, averagely accounting for three fourths of HPV-positive cases (Smith et al., 2009). There are differences regarding the clinicopathological aspects and the pathogenic pathways among vulvar cancers with respect to the status of HPV infection (Del Pino et al., 2013).

Vaccination against HPV is now available for two of the most common high-risk types (HPV16 and HPV18). Infection of HPV16 and/or HPV18 is detected in a majority of HPV-positive vulvar cancer cases. However, there is a variation in the HPV prevalence in different geographic areas. To estimate the potential benefit of HPV vaccine in the prevention of vulvar cancer, the knowledge of HPV prevalence and genotype distribution is of paramount

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importance. There has been very limited data on HPV prevalence and genotype distribution in vulvar cancer from countries in the Southeast Asian region, with only a single previous study from Thailand to our knowledge (De Vuyst et al., 2009; Smith et al., 2009; Del Pino et al., 2013; Ngamkham et al., 2013). That study reported a series of 25 patients of vulvar SCC with rather limited clinicopathological information (Ngamkham et al., 2013).

This study was aimed to evaluate the prevalence of HPV infection and the genotype distribution in vulvar SCC in northern Thailand. We also evaluated the clinicopathological difference with reference to HPV infection in vulvar SCC patients in this region.

Materials and Methods

The study was approved by the institutional ethics committee. The database of the Department of Pathology at the Chiang Mai University Hospital was searched for cases of vulvar SCC which were diagnosed between January 2006 and December 2012 and had available paraffin blocks. Histologic slides of each case were reviewed by a pathologist (S.S.) to confirm the diagnosis of invasive SCC and to select the paraffin blocks for HPV detection. Each paraffin block was matched with the corresponding histologic slide and the area in the block which contained only SCC was marked. The parts of the block which did not contain invasive carcinoma were trimmed off to obtain only the component of SCC in the sample submitted for HPV DNA analysis.

For DNA extraction, 25 μm of material (5 \times 5- μm sections) was cut from each paraffin block. To avoid cross-contamination between tissue samples, the surface area in the microtome was treated after each tissue cutting by using 20% Clorox bleach, and then rinsed and dried with mild detergent, distilled water, and absolute ethanol. After deparaffinization in xylene and rehydration in ethanol, DNA was extracted using a QIAamp Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. A final volume of 40 μl DNA elute was stored at -20°C prior to the polymerase chain reaction (PCR). All samples were screened for the first PCR amplification with primers MY09/MY11 located within the HPV L1 gene. The amplified DNA samples were re-amplified using primers GP5+ and GP6+ as previously described (Siriaunkgul et al., 2008). The adequacy of the specimens was examined by co-amplification of 199-bp fragment of the human β -globin gene in the first-round PCR. Samples that tested negative with primers GP5+/6+ were regarded as negative for HPV DNA. Samples that were positive for HPV DNA were then analyzed by the Linear Array HPV Genotyping Test (Roche Molecular System Inc., Branchburg, NJ), with modification for the use of paraffin-embedded tissue as previously described (Siriaunkgul et al., 2008). The Linear Array Genotyping Test is designed to identify 37 HPV genotypes including 14 high-risk HPV (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) simultaneously in a single assay. To increase the capacity of genotyping, samples that were negative by the Linear Array Genotyping Test were further tested with type-specific PCR targeting the

E6/E7 region in the viral genome of HPV16, HPV18, and HPV52 (Gravitt et al., 2003), which are the 3 most common genotypes in cervical SCC in northern Thailand (Siriaunkgul et al., 2008). The samples that tested positive with primers GP5+/6+ but negative with both the Linear Array Genotyping Test and the type-specific PCR were considered to have an undetermined HPV type. The HPV genotypes were classified based on the previously described data as low-risk, high-risk, probably high-risk, and undetermined-risk (Skapa et al., 2007).

The histologic slides of tumors were reviewed by another pathologist (S.K.) for the histologic subtype and grade of SCC and related lesions, without the knowledge on HPV status and genotyping. The histologic subtype of vulvar SCC was classified according to the 2003 World Health Organization Classification as keratinizing, non-keratinizing, basaloid, warty, or verrucous subtype (Wilkinson and Teixeira, 2003) (Figure 1). The histologic grade was classified into well-, moderately, and poorly differentiated categories (Benedet et al., 2000). The presence or absence of adjacent vulvar intraepithelial neoplasia (VIN) or lichen sclerosus was evaluated only when the specimens included epithelial surface area extending for at least 5 mm away from the last foci of invasive carcinoma. VIN was classified into usual type, which comprised basaloid or warty type of VIN 3, and differentiated type (Wilkinson and Teixeira, 2003) (Figure 2). The status of lymph node metastasis was abstracted from surgical pathology report. The clinical data were obtained from medical records. The tumor stage was classified based on the clinical and pathological data using the 2009 revised International Federation of Gynecology and Obstetrics (FIGO) staging system (Pecorelli, 2009).

The data were analyzed using Intercooled Stata Software Version 11.0 (Stata Corp, College Station, TX, USA). Comparison for the difference of the values of interest was tested by exact probability (Fisher exact) test or T test as appropriate. *p* value <0.05 was considered to be statistically significant.

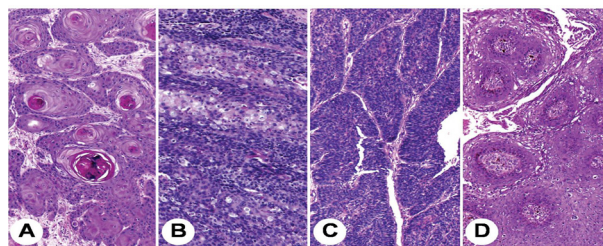


Figure 1. Subtypes of Squamous Cell Carcinoma of the Vulva. A) Keratinizing subtype, B) Non-keratinizing subtype, C) Basaloid subtype, and D) Warty subtype

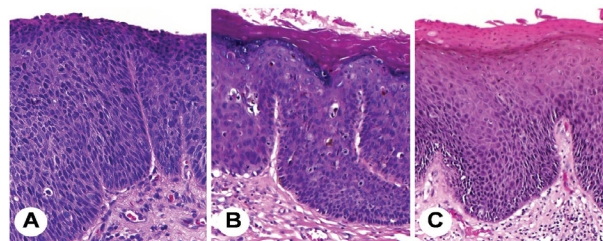


Figure 2. Types of Vulvar Intraepithelial Neoplasia. A) Basaloid type, B) Warty type, and C) Differentiated type

Results

There were 47 cases of vulvar SCC included for HPV analysis in this study. The patients' mean age was 57.9±13.2 years (range 31-82 years). The distribution of FIGO tumor stage was stage I in 25 patients (53%), stage II in 5 patients (11%), stage III in 12 patients (26%), and stage IV in 5 patients (11%). The histologic subtype was classified as keratinizing in 26 cases (55%), non-keratinizing in 8 cases (17%), basaloid in 4 cases (9%), and warty in 9 cases (19%). The histologic grade was classified as well-differentiated in 33 cases (70%), moderately differentiated in 12 cases (26%), and poorly differentiated in 2 cases (4%).

HPV infection was present in 29 cases (62%), whereas the remaining 18 cases (38%) were HPV-negative. All HPV-positive cases with known genotypes had single HPV infections. Among 29 HPV-positive cases, high-risk HPV genotypes (HPV16 and HPV58) were identified in 24 cases (83%), and probably high-risk genotype (HPV26) in 1 case (3%). Low-risk HPV genotype (HPV89) was detected in 2 cases (7% of HPV-positive cases). The remaining 2 HPV-positive cases (7%) had undetermined genotype. HPV genotypes were identified with the Linear Array assay in 21 and type-specific PCR in 6 cases.

Table 1 shows the relationship between patients' mean age and the status of HPV infection and HPV genotypes in all 49 patients. The patients with HPV-positive SCC had a significantly younger mean age than those with HPV-negative tumors (52.7 years vs 66.2 years, $p<0.001$). The patients with HPV16 infection also had a highly significant age difference compared with those with HPV-negative SCC ($p<0.001$).

Table 2 shows a clinicopathological comparison between HPV-positive SCC and HPV-negative SCC patients. Most of the patients (72%) in HPV-positive group were aged 60 years or younger, whereas most of the patients (72%) in HPV-negative group were older than 60 years ($p=0.006$). There was no significant difference in the stage distribution with regard to the status of HPV infection, although the proportion of FIGO stage III-IV patients in HPV-positive group tended to be higher than that of HPV-negative group (41% vs 28%, $p=0.533$). Among 33 cases with inguinal lymph node dissection performed, nodal metastasis was more frequently observed in HPV-positive cases than in HPV-negative cases, but the difference was not significant (41% vs 25%, $p=0.465$).

Table 1. Mean Age in Patients with Vulvar Squamous Cell Carcinoma Stratified by the Status of HPV Infection and HPV Genotypes

| HPV status and genotype | No. (%) | Mean age±SD (years) |
|-------------------------|----------|---------------------|
| HPV-positive | 29 (62) | 52.7±12.5 * |
| HPV16 | 23 (49) | 52.1±12.3 * |
| HPV26 | 1 (2) | 43 |
| HPV58 | 1 (2) | 71 |
| HPV89 | 2 (4) | 59.0±18.4 |
| Undetermined genotype | 2 (4) | 48.5±10.6 |
| HPV-negative | 18 (38) | 66.2±9.9 |
| Total | 47 (100) | 57.9±13.2 |

*p value <0.001 versus HPV-negative cases

Table 2. Comparison of Clinical and Pathologic Characteristics in Patients with Vulvar Squamous Cell Carcinoma Stratified by the Status of HPV Infection

| | | HPV- ve+ (n=29) (%) | HPV- ve- (n=18) (%) | p value |
|-------------------------------|---------------------------|------------------------|------------------------|---------|
| Age | <60 years | 21 (72) | 5 (28) | 0.006 |
| | >60 years | 8 (28) | 13 (72) | |
| Stage | I | 15 (52) | 10 (56) | 0.638 |
| | II | 2 (7) | 3 (17) | |
| | III | 8 (28) | 4 (22) | |
| | IV | 4 (14) | 1 (6) | |
| Histologic subtype | Keratinizing | 12 (41) | 14 (78) | 0.100 |
| | Non-keratinizing | 6 (21) | 2 (11) | |
| | Basaloid | 3 (10) | 1 (6) | |
| | Warty | 8 (28) | 1 (6) | |
| Histologic grade | Well-differentiated | 18 (62) | 15 (83) | 0.336 |
| | Moderately differentiated | 9 (31) | 3 (17) | |
| | Poorly differentiated | 2 (7) | 0 (0) | |
| Adjacent VIN (n=43)* | Usual-type VIN | 16 (62) | 1 (6) | <0.001 |
| | Differentiated-type VIN | 0 (0) | 4 (24) | |
| | Absent | 10 (38) | 12 (71) | |
| Lymph node metastasis (n=33)* | Present | 7 (41) | 4 (25) | 0.465 |
| | Absent | 10 (59) | 12 (75) | |

*Histological review based on the cases with available tissue (adjacent epithelial surface >5mm or lymph node dissection specimens)

Regarding the histologic subtypes of SCC, the overall distribution was not significantly different between HPV-positive group and HPV-negative group ($p=0.100$) (Table 2). However, the difference in proportion of HPV-positive cases was significantly observed between keratinizing subtype (12 of 26 cases or 46%) and the other subtypes combined (17 of 21 cases or 81%, $p=0.019$), and between keratinizing subtype (46%) and warty subtype (8 of 9 cases or 89%, $p=0.048$). HPV-negative SCC tended to be more frequently well-differentiated than HPV-positive tumors (83% vs 62%, $p=0.191$). Among 24 cases with high-risk HPV genotypes, 14 cases (58%) had well-differentiated SCC, while both cases with low-risk HPV genotype had well-differentiated tumors.

The presence of VIN adjacent to SCC was more common in HPV-positive cases than in HPV-negative group (62% vs 29%, $p=0.062$). There was a significant difference in the distribution of the types of VIN between HPV-positive and HPV-negative cases (Table 2). VIN of usual type (basaloid or warty) was present in 62% of HPV-positive cases, which was significantly more frequent than that in HPV-negative cases (6%, $p<0.001$). Meanwhile, differentiated-type VIN was present in 24% of HPV-negative cases, but was not observed in any HPV-positive cases ($p=0.019$). Lichen sclerosis was seen adjacent to carcinoma in 1 HPV-negative case, and squamous hyperplasia was observed in another 2 cases (HPV-positive and HPV-negative in each).

Discussion

Vulvar cancer is rather uncommon in gynecologic oncology practice. However, there is some variation in the incidence of vulvar cancer which appears to be geographically dependent. Considering the data from

different regions in the world which include at least 50 cases per site, the incidence of vulvar cancer is lowest in the East Asian region (China, Japan, and Korea) with the age-standardized incidence rate (ASR) of 0.2-0.3 per 100,000 women, whereas higher incidence rates are reported from the countries in Europe and America (ASR 1.2 to 2.2) (Curado et al., 2007). In Southeast Asia, the ASR of vulvar cancer in the region where this study was undertaken (Chiang Mai, Thailand) was 1.2, which was higher than that reported from the other sites (0.3 to 0.7) (Curado et al., 2007). It should be noted that the higher ASR of vulvar cancer in our region seems to parallel with the ASR of cervical cancer as Chiang Mai also had the highest ASR (28.9) among Southeast Asian countries (Curado et al., 2007). However, the incidence rate of vulvar cancer does not always correlate with that of cervical cancer in the other regions such as Uganda and Zimbabwe, where there is a very high incidence of cervical cancer (ASR 45.8-47.3) but a rather low incidence of vulvar cancer (ASR 0.3-0.7) (Curado et al., 2007). The explanation for geographic variation of the incidence of vulvar cancer remains unclear, although this could be partly due to heterogeneous etiologies of vulvar cancer (van der Avoort et al., 2006). The incidence of vulvar cancer in our region was only slightly lower than the overall ASR of 1.4 in the United States (SEER, 14 registries) and in Canada (Curado et al., 2007).

Recent information in the literature indicates that there are 2 different pathogenic pathways of vulvar SCC, one is associated with HPV infection whereas the other is not (Del Pino et al., 2013). In the HPV-associated pathway, vulvar SCC evolves from usual-type VIN (van der Avoort et al., 2006). In the other pathway unassociated with HPV infection, vulvar SCC is mostly of keratinizing type and is associated with p53 mutations (Pinto et al., 2010; Del Pino et al., 2013). HPV-negative SCC is also associated with differentiated-type VIN or lichen sclerosus (Pinto et al., 2010; Alonso et al., 2011). Almost one half of cases with keratinizing SCC were HPV-positive in our study similar to the finding in a previous report from the United States (Gargano et al., 2012). In previous studies, the prevalence of HPV in keratinizing SCC (range 6% to 49%) was lower than that of the other subtypes of vulvar SCC which ranged from 70% to 92% (De Vuyst et al., 2009; Smith et al., 2009; Gargano et al., 2012; de Sanjose et al., 2013). However, there is still an overlapping spectrum of subtypes between HPV-positive and HPV-negative SCC, and this causes difficulty in the prediction of HPV status based on the morphology of SCC alone (Del Pino et al., 2013). In the present study, there were significant differences between the HPV-positive SCC and the HPV-negative SCC in the proportions of usual-type VIN ($p < 0.001$) and differentiated-type VIN ($p = 0.019$), which is in keeping with the different pathogenic pathways between both groups (Del Pino et al., 2013).

In most studies, a difference in the age distribution of vulvar SCC patients was observed between HPV-positive group and HPV-negative group; the former group was generally at least 5-10 years younger than the latter (Skapa et al., 2007; Sutton et al., 2008; Alonso et al., 2011). In the present study, a highly significant difference in patients'

mean age with regard to the presence of HPV infection was noted. The younger patient age in HPV-positive patients was apparently observed in the patients with high-risk HPV infection. The patients with HPV infection of low-risk or undetermined genotype also tended to have a younger mean age than those with HPV-negative tumors, although the difference was not significant.

In the present study, the prevalence of HPV infection in vulvar SCC was 62% in northern Thailand. This prevalence is higher than that reported in the previous smaller series from Thailand (44%) (Ngamkham et al., 2013), but is comparable to the HPV prevalence range of 59% to 70% reported in the vulvar cancer studies from North America (Insinga et al., 2008; Sutton et al., 2008; De Vuyst et al., 2009; Smith et al., 2009; Gargano et al., 2012). There appears to be a geographic variation in the prevalence of HPV infection in vulvar SCC. The HPV prevalence in this study and in the studies from North America was higher than that reported from East Asia (38%), Europe (33-35%), Oceania (29%), and South America (24%) (De Vuyst et al., 2009; Smith et al., 2009). The difference may be partly related to the types of specimen used in each study, the variation in the distribution of SCC subtypes, or the variation in HPV detection techniques (Smith et al., 2009; Gargano et al., 2012; de Sanjose et al., 2013). The latter possibility is supported by cervical cancer studies where an increase in HPV detection rate may link to recent advances in HPV detection systems (Li et al., 2011). However, it should be noted that the regional difference in HPV prevalence between North America and Europe could still be observed in the recent studies using the current sensitive HPV detection assays (50-70% vs 15-35%, respectively) (Riethdorf et al., 2004; Sutton et al., 2008; Kowalewska et al., 2010; Alonso et al., 2011; Gargano et al., 2012; de Sanjose et al., 2013).

HPV16 is the predominant genotype in HPV-positive vulvar SCC with prevalence ranging from 73% to 80% in previous reports (Insinga et al., 2008; De Vuyst et al., 2009; Smith et al., 2009; de Sanjose et al., 2013). In our study, HPV16 was detected in 79% of HPV-positive cases or 49% among all vulvar SCC cases. High-risk genotypes other than HPV16 are uncommon in vulvar SCC. In a meta-analysis, HPV16 accounted for 32.2% of vulvar cancer, HPV33 in 4.5%, and HPV18 in 4.4% (De Vuyst et al., 2009). HPV33 and HPV18 were not detected in vulvar SCC in the present study, whereas, in the previous study from Thailand, HPV33 was identified in 2 of 25 cases (8%) and HPV18 in 1 case (4%) (Ngamkham et al., 2013). In the present study, infection of multiple HPV genotypes was not observed, whereas the rate of multiple HPV infections was reported to be 2.8% in the meta-analysis study (De Vuyst et al., 2009). Higher rates of multiple HPV infections (5.8 to 12.1%) was observed in the studies from the United States (Insinga et al., 2008; Sutton et al., 2008) and the previous study from Thailand (4 of 25 cases, 16%) (Ngamkham et al., 2013). Infection of low-risk HPV genotype (HPV89) was found in 4% of vulvar SCC in the present study, which was comparable to the estimated rate of 3.6% in pooled cases from the studies in the United States (Insinga et al., 2008). Among the cases with low-risk HPV genotypes in pooled data from

previous studies, HPV6 was the most common, followed by HPV11 (Insinga et al., 2008; Smith et al., 2009).

The mechanism by which HPV causes vulvar carcinoma may be similar to the carcinogenic role of HPV in cervical cancer (van der Avoort et al., 2006). High-risk HPV induces neoplastic transformation of epithelial cells through binding of E6 and E7 viral oncoproteins to important tumor suppressor gene products that regulate cell cycle; p53 and retinoblastoma (Rb) proteins (Pinto et al., 2010). Binding of E6 to p53 protein leads to p53 degradation, while binding of E7 to Rb protein leads to Rb inactivation (van der Avoort et al., 2006). The deregulation of cell cycle is followed by an abnormal expression of cell cycle-associated proteins (Pinto et al., 2010), and immunohistochemical overexpression of p16 protein is typically observed in HPV-positive vulvar SCC and usual-type VIN similar to cervical SCC (Alonso et al., 2011). Although the pathogenesis of vulvar SCC associated with "low-risk" HPV genotypes has not been clearly defined (Del Pino et al., 2013), a recent study has confirmed the role of these viruses in the carcinogenesis of occasional anogenital carcinomas and has suggested heterogeneous carcinogenic pathways among different genotypes of low-risk HPV (Guimera et al., 2013).

In previous studies, there were conflicting results in the prognostic difference in vulvar SCC with regard to the HPV status (Del Pino et al., 2013). Among the recent large studies with approximately 100 or more cases of vulvar SCC, van de Nieuwenhof et al. (2009) reported a worse disease-specific survival in vulvar SCC associated with differentiated-type VIN, which is mostly HPV-negative. In another study, Sutton et al. (2008) found that vulvar SCC patients with high-risk HPV genotypes had a lower rate of lymph node metastasis than those with HPV-negative tumors (odds ratio 0.28, 95% confidence interval 0.09-0.89). This finding suggests a worse prognosis in HPV-negative group because lymph node metastasis was found to be an independent prognostic variable in vulvar SCC (van der Steen et al., 2010; Alonso et al., 2011). On the contrary, Pinto et al. (2004) did not observe a prognostic difference based on the HPV status. Alonso et al. (2011) also found no significant survival difference between the HPV-positive group and the HPV-negative group, although the 5-year disease-free survival tended to be lower in the HPV-positive group (39.8% vs 49.8%). In the present study, HPV-positive patients tended to have a higher rate of lymph node metastasis, and a higher FIGO stage than the HPV-negative group. Survival analysis was not performed in this study because of the limited follow-up duration which may be too short to obtain a meaningful analysis.

In conclusion, HPV infection was detected in 62% of vulvar SCC in northern Thailand and HPV16 was the predominant genotype, similar to the data reported from other regions. HPV-positive SCC occurred in younger patients compared with HPV-negative SCC, and was associated with usual-type VIN. Vaccination against HPV16/18 may potentially prevent almost one half of vulvar SCC in northern Thailand.

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