RESEARCH ARTICLE

Preliminary Study on Human Papillomavirus Frequency and Specific Type-distribution in Vulva Cancer from Thai Women

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Abstract

Vulva cancer is rare among all gynecological cancer worldwide, including Thailand, and mainly affects older women. Persistent high risk type infection of human papillomavirus (HPV) is the one important factor for developing cancer. In this study, we focused on HPV DNA investigation and type-specific distribution of HPV in 25 formalin-fixed paraffin-embedded (FFPE) samples collected from Thai women with vulva cancer histologically confirmed by the National Cancer Institute, Thailand, during 2003-2011. HPV DNA detection and genotyping were undertaken with polymerase-chain reaction and enzyme-immunoassay using GP5+/bio6+ consensus specific primers and digoxigenin-labeled specific oligoprobes, respectively. Human β-globin genes was used as the internal control. Our results showed that 44% (11/25) of all vulva cancer samples were HPV-positive. All of them are high risk HPV type infection, detected as single (63.64%, 7/11) and/or double infections (4/11, 36.36%). HPV 16 was the most common type identified in vulva cancer, followed by HPV 35, 33, 18 and 58. In conclusion, this study presented that HPV-16 is observed at the highest frequency in this cancer, similar to cervical cancer, with HPV 18 being less frequent. Although the sample size was small and could not represent overall incidence and prevalence in Thai women, these preliminary data for vulva cancer are of interest since they reinforce the necessity for HPV screening or vaccination in Thailand.

Keywords: Human papillomavirus - frequency - type distribution - vulva cancer - Thai women

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Introduction

Vulva cancer is a rare disease, representing about 3-5% of all gynecological cancer among women around the world (Parkin, 2006; van de Nieuwenhof et al., 2008; Dittman et al., 2011). The worldwide age standardized incidence rate (ASR) of vulva cancer lie between 0.5-1.5 per 100,000 (Shukla et al., 2009) and approximately 60% of all vulva cancer that accounted for 26,800 cases of which 15,700 was found in developed countries (Sankaranarayanan et al., 2006; WHO/ICO, 2010). Vulva cancer is the important cancer in women, which could not be ignored, although the incidence of this cancer is low and limit of epidemiological data. Vulva cancer is typically affects in older women (Giuliano et al., 2008). It usually observed the high prevalent in women over 65 years (Karunaratne, 2011). From the survey of cancer in Thailand during 2004-2005 report, the rate of vulva cancer was the low incidence among Thai women, with ASR of 0.4 per 100,000 women (Khuaprema et al., 2012). Numerous studies are often reported epidemiological data of vulva cancer parallels with the other gynecological cancer such as cervical, vagina etc, including, HPV infection, a history of sexual behavior, smoking, genital wart and other sexual transmitted disease (Trimble et al., 1996; Madeleine et al., 1997).

The most common vulva cancer cases are squamous cell carcinoma, followed by melanoma, basal cell carcinoma and adenocarcinoma (WHO/ICO, 2010; Karunaratne, 2011). The majority of invasive squamous cell carcinoma of vulva can be classified as two important types, the first one is a differentiated keratinizing squamous cell carcinoma, which is common type mainly occur in elderly women and significantly arise in women within a background of non-neoplastic epithelial disorders (eg. lichen sclerosus) and often differentiated vulva intraepithelial neoplasia (Differentiated VIN type; dVIN) (Yang et al., 2000; Fox et al., 2003; Jones et al., 2004; Ruhul et al., 2005; Hampl et al., 2007; van de Nieuwenhof et al., 2008; Terlou et al., 2010) and only 4-21% are reported to be associated with HPV infection (Hording et al., 1994; Trimble et al., 1996; Madeleine et al., 1997; van de Nieuwenhof, 2008). The second type is classified as non-keratinizing carcinoma that are related with warty, basaloid or mixed types or mixed types between warty and baseloid VIN (identified as Usual VIN; uVIN) and

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typically affect in younger women. More than 80% cases of squamous cell carcinoma with non-keratinizing and usual vulva intrapithelial neoplasia (uVIN) patients were infected with high-risk HPV type, predominantly HPV type 16 (Monk et al., 1995; Madeleine et al., 1997; van de Avoort et al., 2006; van de Nieuwenhof et al., 2008; Smith et al., 2009; Terlou et al., 2010; Karunaratne, 2011). Human papilomavirus is a small virus that is the etiological agent to cause a wide range of genital lesion, non-genital epithelial lesion and cancer cells (zur Haussen, 2002; Ramet, 2010). Genital HPV infection is the important risk factor of the most prevalent sexually transmitted infections worldwide (Walboomers et al., 1999). Of the 15 HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73 and -82) are classified as high-risk or oncogenic type, have been found to be more associated with malignancy cells develpment (Shukla et al., 2009) such as intraepithelial neoplasia or cancer cells of cervical, vagina, panis, anal or vulva (Ramet et al., 2011).

The purpose of this study was to investigate frequency of HPV infection and type-distribution in vulva cancer of Thai women which were diagnosed during 2003-2011 at Pathology Department, National Cancer Institute, Thailand.

Materials and Methods

Samples recruitment and preparation

Twenty-five formalin-fixed and paraffin-embedded (FFPE) tissues of vulva cancer, which were collected, diagnosed during 2003-2011 with histologically confirmed, were obtained from Specimen Bank and Pathology Department, National Cancer Institute. This protocol study was approval by Research and Ethic committee of National Cancer Institute, Thailand.

Three of 5-10 μM thickness sections of each sample FFPE block were cut and collected in sterile vial. The contaminant between samples were eliminated by changing of groves, carefully cleaning the microtome, blade replacement before cutting the next sample and using the sterile forceps to transfer tissue sections to their sterile vial. Tissue samples underwent deparaffinization with xylene, twice rehydration in analytical-graded absolute ethanol, and then carefully discharge the remaining ethanol. All of samples were dried in the room temperature or heat-block at 37°C to ensure that no residual absolute ethanol.

DNA extraction

Genomic DNA was extracted from dried tissues using QIAamp DNA Mini kit (QIAGEN, England) according to manufacturer's instructions and stored at -20°C for further analysis. The quality of DNA was evaluated by polymerase-chain reaction with β-globin, house keeping gene, primers (Jacob et al., 1996): β-globin sense 5-ACA CAA CTG TGT TCA CTA GC-3 and β-globin antisense 5-GAA ACC CAA GAG TCT TCT CT-3. PCR was performed in a total volume of 25μl, containing 5 μl extracted DNA, 0.4 μM of each specific primer, 2.5U Taq polymerase, 200nM of each dNTPs and 1.5mM $MgCl_2$ and the amplification was performed using the GeneAmp 9700 thermal cycler (Applied Biosystemic, USA). The PCR cyclic condition under the following; initial denaturation step of 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The last step was final extension of 5 min at 72°C. The presence/absence band specific for human β-globin was visualized on 2% agarose gel electrophoresis by transilluminator (SYNGENE, England).

HPV DNA detection and genotypes

The samples which showed the β-globin positive were continued investigate HPV DNA by using GP5+ (5-TTT GTT ACT GTG GTA GTA ACT AC-3) and biotin GP6+ (5-GAA AAA TAA ACT GTA AAT CAT ATT C-3) specific consensus primers to detect HPV DNA and genotypes (Jacob et al., 1997). A 50 μl total volume of PCR mixture was prepared by adding 10 μl extracted DNA, 0.4 μM of each specific primer, 2.5U Taq polymerase, 200nM of each dNTPs and 1.5mM $MgCl_2$ and PCRs condition were run following profile of initial denature at 94°C for min, followed by 40 cycles of 94°C for 1 min, 38°C for 2 min and 72°C for 1.30 min. The finally step was re-extension at 72°C for 4 min. HPV DNA detection and genotype were under taken by using Enzyme-immunoassay (EIA) method with two HPV oligoprobe cocktail that categorized as high risk group cocktail (consist of HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 59, 66, 56, 58, 68) and low risk group (consist of HPV type 6, 11, 26, 34, 40, 43, 42, 44, 53, 54, 55, 57, 61, 70, 71, 72, 73, 81, 82, 83, 84, 89). Specific probe sequences and procedures are described by Jacob et al. (1997; 2000). Briefly, a total volume of 5μl PCR products and 50 μl freshly prepared were added to each well of streptavidin-coated micro-titer plate (Roche, Germany) and incubated at 37°C for 60 min to bind biotinylated compounds. Unbound biotinylated PCR products were removed by three times washing with freshly prepared 1xSCC, 0.5% Tween 20 mixed solution. A cocktail or specific oligonucleotide probes which were labeled with digoxigenin was added and incubated at 37°C for 60 min to allow hybridization and then unbound was eliminated by three times washing. Detection of specifically bound digxigenin-labeled probe was achieved by adding conjugate solution (Roche, Germany) and substrate (p-Nitrophenyl phosphate; pNpp) (sigma, USA), respectively. The optical density, 405nm and 620nm, was measured at 60 min and 16-24 hours (Jacob et al., 1997).

Results

This study investigated the HPV frequency and type-distribution in 25 samples from formalin-fixed and paraffin-embedded (FFPE) tissues of Thai women with vulva squamous cell carcinoma, age range 34-91 years (mean 65.28 years, SD±13.78). Fifteen and ten FFPE tissue samples were obtained from specimen bank and Pathology Department, National Cancer Institute, respectively. Forty percent (10/25) cases were younger than 65 years old, whereas 60% (15/25) cases were equal to/older than 65 years old.

Polymerase chain reaction using GP5+/bio6+ specific consensus primers and Enzyme immunoassay revealed 11

of 25 (44%) samples as positive for HPV infection, which 7/10 samples were detected in the first group (younger than 65 years) and 4/15 samples were found in the second group (equal to/older than 65 years), as shown in Figure 1.

All of HPV positive samples had to least one high-risk type infection and low risk type infection did not found in this study. HPV 16 was the most frequent type, follow by HPV 35, 33, 18 and 58. In samples where HPV positive, we could be detected HPV types both of single and double infection. The majority of vulva squamous cell carcinoma samples in this study contained a single HPV types (7/11, 63.64%), as shown in Figure 2 and Table 1. whereas 4 of 11 (36.36%) samples were found to have double infection such as HPV 16/18, HPV 16/33, HPV 16/35 and HPV 35/58, as shown in Table 1.

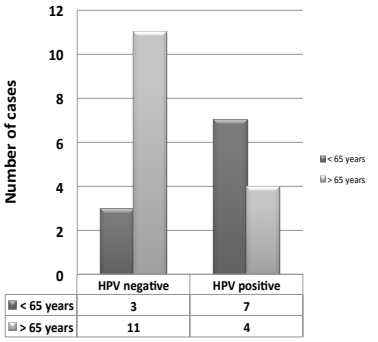

Figure 1. Human papillomavirus DNA Detection in Vulva Squamous Cell Carcinima Tissues, Considerable Divided in Two Main Groups, <65 years (n=10) and >5 years (n=15)

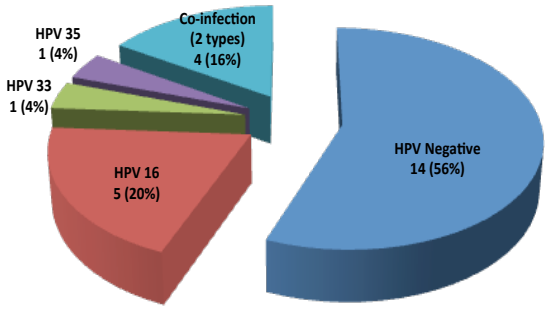

Figure 2. Overall of Human papillomavirus Type-distribution in Vulva Squamous Cell Carcinoma Tissues from Thai Women (n=25) Squamous Cell Carcinoma Tissues from Thai Women (n=25)

Table 1. Specific Type-distribution of Human Papillomavirus in 25 FFPE Sample Tissues from Thai Women with Vulva Squamous Cell Carcinoma (n=25)

	Frequency (%)[a]	Percent of HPV positive
HPV negative	**14 (56)**	
HPV positive	**11 (44)**	**100**
Single infection	**7**	**63.63**
HPV 16	5	45.45
HPV 33	1	9.09
HPV 35	1	9.09
Double infection	**4**	**36.36**
HPV 16 and HPV 18	1	9.09
HPV 16 and HPV 33	1	9.09
HPV 16 and HPV 35	1	9.09
HPV 16 and HPV 58	1	9.09

[a]percent of total samples (n=25)

Discussion

Vulva cancer is a rare cancer in women worldwide; the higher incidence rate is related with increase age (Dittmer et al., 2011). Numerous reports, indicated that the persistent HPV infection was the most important causes for the development of cervical cancer and other genital tract, up to 90% of high-grade cervical intraepithelial lesion and cancer were associated with HPV infection (Walboomers et al., 1999; zur Hausen, 2002; Ramet et al., 2010), but not all of vulva cancer cause that are induced by HPV, it depend on the group of population, age, life style etc in different geographic areas (Hampl et al., 2007). The high incidence of non-keratinizing (basaloid/warty) carcinoma has been easily detected HPV infection and primarily affect in young women, whereas the keratinizing carcinoma of older women is rarely HPV related and often develops in women with a background of non-neoplastic epithelial disorder such as lichen sclerosus (LS) and often differentiated intraepithelial neoplasia (dVIN) (Fox et al., 2003; Yang et al., 2003; Ruhul, 2005; Hampl et al., 2007; van de Nieuwenhof, 2008; Gil-Prieto et al., 2011).

In this study was focused on the HPV infection frequency and type specific distribution of 25 vulva squamous cell carcinoma samples from Thai women, which was indicated that the women with below 65 years was found HPV infection (70%, 7/10) higher than the women with equal to/older than 65 years (26.6%, 4/15). It suggested that, HPV infection may play an important role in the pathogenesis of vulva carcinoma among younger women (Madeleine et al., 1997). All of HPV positive was infected with high-risk types; 63.64% (7/11), that was represented as single type infection and 36.36% (4/11) was showed as double infection. Interestingly, low-risk HPV type such as HPV 6 or HPV 11 did not detect in this study, suggested that all of sample tissues were selected from vulva cancer tissues, not wart or benign tumor. And HPV 16 was the predominant HPV type that found in both groups. Approximately 72% (8/11) of HPV positive was counted as HPV16, while HPV 18 was observed only 9.09% (1/11) as same a meta-analysis investigation of HPV prevalence in vulva, vagina and anal from 93 studies which was conducted by De Vuyst et al. (2009), indicated that HPV 16 was more frequently (>75%) and HPV 18 showed less frequently (<10%) in HPV positive of vulva carcinoma.

The five common frequent HPV types that could be detected in this study were HPV-16, -35, -33, -18 and -58, which have similar pattern with the other reported, as compare to the recent study by Baldez et al. (2012), the four most common HPV types of vulvar lesions from Brazil gynecological patients could be identified as HPV 16, 31, 33 and 18. Almost of single type infection was HPV 16, whereas HPV 18 could be detected as co-infection with the other high-risk types. And the reported by Remet et al. (2010) and Munger et al. (2004), represented that HPV 16, 18, 31 and 33 were also common found in cancer of

vulva in their studies.

However, there are some limitations of this study such as the low incidence of vulva cancer and approximately 10% of cases were referred from the other Cancer Center to diagnosis confirmed, that lead to lose some patient's information and histopathology. In addition to the tiny sample tissues were difficult to cut by bladder and minimal volume of DNA from FFPE extraction was not enough to analyze, the increased numbers and volume of tissue samples can be dissolved this problem. Furthermore, the population group, sample size and the variety of age cut off, which was used to categorize the younger or older women group, are to be considerable. They are the important points that may be indicated the different data reported of HPV infection in vulva cancer (Hampl et al., 2006; Giuliano et al., 2008; Ramet et al., 2010). In our study, 63.64% (7/11) of HPV-positive could be detected in women with below 65 years compared to those 85% in women younger 50 years of age in the other study (Hampl et al, 2006; Ramet et al, 2010).

In conclusion, this study indicated that HPV 16 was the most common HPV type present in Vulva carcinoma from Thai women as same as cervical cancer, although the sample size was small and did not represent overall incidence and prevalence of population, but it is very interesting and baseline data of this cancer. HPV infection is the effective factor that causes the development of abnormality tissues and carcinoma of genital tract. Thus, the efficiency prevention programs which reinforce the HPV screening and HPV vaccination, including the update technologies for HPV detecting /testing and disseminate knowledge to the public awareness for primary prevention in cancer may decrease the incidence of HPV-infection disease in the future.

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