ความชุกของการติดเชื้อ Human Papillomavirus ในเนื้อเยื่อมะเร็งรังไข่ชนิดเยื่อบุผิวของผู้ป่วยทางภาคตะวันออกเฉียงเหนือของประเทศไทย

Prevalence of Human Papillomavirus Infection in Epithelial Ovarian Tissues of Patients in the Northeastern Region of Thailand

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บทคัดย่อ

บทบาทของเชื้อไวรัสฮิวแมนปาปิลโลมา (เอชพีวี) ในมะเร็งรังไข่ยังคงมีข้อถกเถียงกันอยู่ การศึกษานี้จึงได้ตรวจหาการติดเชื้อเอชพีวีในเนื้อเยื่อรังไข่ที่ผ่านการ embedded อยู่ในพาราฟินบล็อก กลุ่มตัวอย่างประกอบด้วยเนื้อเยื่อรังไข่ปกติ เนื้องอกรังไข่และมะเร็งรังไข่ชนิดเนื้อเยื่อบุผิวจากผู้ป่วยในภาคตะวันออกเฉียงเหนือของประเทศไทย และประเมินความสัมพันธ์ระหว่างการติดเชื้อเอชพีวีและลักษณะทางจุลพยาธิวิทยาของชิ้นเนื้อ ดีเอ็นเอที่สกัดถูกตรวจหาเอชพีวีและไทป์ของเชื้อไวรัส โดยใช้เทคนิคเรียลไทม์พีซีอาร์และ reverse line blot hybridization ตามลำดับ ผลการศึกษาพบว่าความชุกของเอชพีวี ในกลุ่มตัวอย่างมะเร็งรังไข่ (ร้อยละ 32) พบได้สูงกว่าที่พบในรังไข่ปกติ (ร้อยละ 25) และ เนื้องอกรังไข่ (ร้อยละ 26) ซึ่งไม่แตกต่างกันอย่างมีนัยสำคัญ พบการติดเชื้อไวรัสชนิดมีความเสี่ยงสูงไทป์ 16 ได้บ่อยที่สุด แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญระหว่างกลุ่มตัวอย่าง พบแบบแผนการติดเชื้อของเอชพีวีไทป์เดียวและแบบผสม (สองไทป์หรือมากกว่า) ได้ในทุกกลุ่มตัวอย่าง โดยพบแบบแผนการติดเชื้อเอชพีวีแบบผสมได้มากกว่าชนิดไทป์เดียวในกลุ่มเนื้องอกและมะเร็งรังไข่ การศึกษาความสัมพันธ์ระหว่างการติดเชื้อเอชพีวีกับลักษณะทางจุลพยาธิวิทยาพบว่า การติดเชื้อเอชพีวีมีความสัมพันธ์กับเนื้องอกรังไข่ชนิด serous (ร้อยละ 11) และ mucinous (ร้อยละ 12) และพบมากในมะเร็งรังไข่ชนิด serous (ร้อยละ 21) ผลการศึกษานี้แสดงให้เห็นว่ามีการติดเชื้อเอชพีวีร่วมกันหลาย

ไทป์ในเนื้อเยื่อรังไข่ และการติดเชื้อเอชพีวีมีความสัมพันธ์กับการเกิดเนื้องอกรังไข่ชนิด serous และ mucinous และมะเร็งรังไข่ชนิด serous ดังนั้นเอชพีวีอาจมีบทบาทต่อการเกิดเนื้องอกรังไข่และมะเร็งรังไข่บางชนิด

คำสำคัญ: *ฮิวแมนปาปิลโลมาไวรัส มะเร็งรังไข่ชนิดเยื่อบุผิว ภาคตะวันออกเฉียงเหนือของประเทศไทย*

Abstract

The conflicting result of human papillomavirus (HPV) infection in ovarian cancer has been published. The role of HPV in ovarian cancer is still controversial. This study investigated HPV infection in formalin-fixed paraffin-embedded (FFPE) ovarian tissues including normal, benign and cancer in northeastern Thailand and evaluated the relationship between HPV infection and histological finding. The extracted DNA was investigated for HPV infection and HPV genotype using real-time PCR and reverse line blot hybridization, respectively. The results showed that higher HPV prevalence was found in EOC (32%) than in normal (25%), and benign (26%) but was not significantly different. HPV16 was the most common type found in each group. Single and double HPV type infections were frequently found in all groups. Mixed (double/multiple) HPV type infection was found higher than single infection in benign and cancer groups. HPV infections are associated with benign epithelial ovarian tumors, especially mucinous and serous histological subtype and serous subtype of EOC. This result demonstrated that HPV might play a role in some specific subtypes of ovarian tumor and cancer in northeastern Thailand.

Keywords: *Human papillomavirus, Epithelial ovarian cancer, Northeastern Thailand*

1. Introduction

Ovarian cancer (OC) is the sixth most common emerging cancer in Thai women (Wilailak & Lertchaipattanakul, 2016). Nearly 90 percent of OC are epithelial ovarian cancers (EOC), which develop in the tissues outside the ovaries and is sub-classified to subtypes serous, mucinous, clear cell, and endometrioid carcinomas (Desai et al., 2014; Lawrie, Bryant, Cameron, Gray, & Morrison, 2013; Rosen et al., 2009). Serous carcinoma (SC) is the most common subtype of EOC and the sixth largest cause of cancer-participatory death of women in several countries. In the United States and Europe, approximately 22,280 (15,500 deaths) and 69,565 (44,280 deaths) women developed EOC in 2012 and 2008, respectively ("Global Cancer Observatory," n.d.; Siegel, Naishadham, & Jemal, 2012). According to World Health Organization (WHO)'s database-CI5plus reported, the highest incidence of OC was in Europe and Northern America, but the lowest one was in Africa and Asia (Y.-C. Chiang et al., 2013).

The advance stage of OC might be caused by late diagnosis, imperfect surgical debulking, poor general medical strings, and the default of effective screening trials; those will lead to a complicated status of other diseases and a poor prognosis (Wentzensen et al., 2016). Over 70 percent of the EOC patients have the high mortality due to

detection at a late stage (Gubbels, Claussen, Kapur, Connor, & Patankar, 2010). The main risk factors of EOC can be classified as genetics and non-genetic factors. Genetic factors normally come from gene mutations, preferentially BRCA1/BRCA2 (Kurman & Shih, 2010) and hereditary nonpolyposis colorectal cancer. Non-genetic motives are endometriosis and co-morbid diseases (Munksgaard & Blaakaer, 2012). In contrast, previous epidemiological studies proposed that reducing the number of ovulatory cycles like tubal ligation, hysterectomy and using oral contraceptives had been reported to be a risk reduction (Ness et al., 2000; Riman, Nilsson, & Persson, 2004).

Ovarian carcinogenesis is a complicated and multifactorial process that is strongly associated with abnormalities in multiple gene families and related to numerous genetic and epigenetic events (Gadducci, Cosio, Tana, & Genazzani, 2009). Tumor suppressor genes and oncogenes are also involved in ovarian cacinogenesis by changing their normal control of biological pathways. These genetic alterations disturb the functions that include cell cycle control, proliferation, apoptosis, migration, differentiation and other processes, which can lead to the pathological condition of tumor transformation.

Human papillomavirus (HPV) is a small double-stranded DNA virus, which is the most common cause of sexually transmitted infection (STI). More than 200 types of HPV (Sohrabi, Hajia, Jamali, & Kharazi, 2017) have been identified and classified into two groups; oncogenic and non-oncogenic (Castellsagué, Bosch, & Muñoz, 2002). HPV infects cutaneous and mucosal squamous epithelial cells (zur Hausen, 1996). Viral early proteins, E6 and E7 of HPV oncogenic group, sometimes named as high-risk HPV (HR-HPV) can induce transformation of epithelial cells by alteration of some proteins involving in cell cycle (Chong et al., 2010). HPV is associated with approximately 99% of the cervical cancer cases (Walboomers et al., 1999). It is also related to several different cancers of the vulva, vagina, penis, anus, lung, bladder, prostate, tongue, larynx, esophagus, tonsil and nasal cavities (Cho et al., 2017; zur Hausen, 1996). A previous study has reported that HPV plays a role in ovarian cancer (Dadashi et al., 2017) but is still controversial (Wu et al., 2003).

In 1987, HPV detection in ovarian cancers by in situ hybridization (ISH) and immunohistochemistry (IHC) techniques was firstly reported (Gupta, Pilotti, Rilke, & Shah, 1987). Nowadays, the prevalence of HPV infection in EOC has been reported by various research groups. Several studies by different methods showed that the highest prevalence of HPV was 67% whereas some studies could not detect HPV infection in EOC (Rosa et al., 2013; Svahn, Faber, Christensen, Norrild, & Kjaer, 2014). The HPV prevalence is different and mostly evidenced by geographical area. These effects might be involved by the technique used and people's behavior. The difference in sample size was also reported (Dadashi et al., 2017; Rosa et al., 2013; Svahn et al., 2014). Thus, this study aimed to investigate the prevalence of HPV infection in EOC tissues in women in northeastern Thailand by using real time polymerase chain reaction (real-time PCR) and reverses line blot hybridization (RLBH) as well as evaluate the relationship between HPV infection and various histologic subtypes of ovarian cancer.

2. Objectives

To assess the prevalence of HPV infection in EOC female patients in northeastern Thailand and evaluated the relationship between HPV infection and various histologic subtypes of ovarian cancer.

3. Materials and Methods

3.1 Clinical specimens and sample size calculation

Clinical specimens were sourced from the patients, who visited the Gynecology Department, Srinagarind Hospital, Khon Kean University, Khon Kean Province, Thailand from January 2013 to April 2018. The patients, were histologically confirmed as EOC, benign epithelial ovarian tumors, and normal ovarian tissues (uterine leiomyoma, uterine prolapse and adenomyosis, etc.). The Ethic Committee of Khon Kean University in Human Research (HE601399) has approved the study procedure. Sample size calculation from the previous study reported that prevalence of HPV-infected in EOC was 25.0% (Shanmughapriya et al., 2012). The formula for calculation of sample size is $n = [Z^2\alpha_{/2} p (1-p)] / d^2$. A total of 215 formalin-fixed paraffin-embedded (FFPE) samples including: normal (92), benign (57) and cancer (66) were collected.

3.2 DNA extraction from FFPE ovarian tissues

Each FFPE ovarian tissue block was sectioned by an unused knife to prevent contamination. Five to eight of 5 µm sections of each sample were transferred to a 1.5 ml microcentrifuge tube. The sections were deparaffinized, washed three times in 1 ml xylene followed by vortexing and centrifugation at the maximum speed: 13,000 × g, 10 minutes, and then, the supernatant was removed. The pellet of the sample was washed three times in 1 ml of absolute ethanol and 1 ml 1XPBS and then air dried. The DNA extraction was carried out by the steps of Tissue DNA Kit (Omega Bio-tek, Georgia, USA). Briefly, the pellet was incubated with 200 µl TL buffer with 25 µl OB Protease Solution in 55 °C until the tissue lysis was completed. The specimen was centrifuged at the maximum speed for 5 minutes, and the supernatant (not comparable) was transferred to a sterile 1.5 ml microcentrifuge tube. The solution of 220 µl BL buffer was added and incubated at 70 °C of 10 minutes. The entire sample was transferred to the DNA Mini Column into a 2 ml collection tube, then the maximum speed was used for centrifugation: 10,770 rpm for 1 minute at room temperature and the filtrate was discarded. The 500 µl HBC buffer was added into the column that was centrifuged at maximum speed for 30 seconds, then the filtrate and the collection tube were removed. The DNA Mini Column was inserted into a new collection tube. The 700 µl DNA wash buffer was added into the column and centrifuged at the maximum speed for 30 seconds, then the filtrate was discarded. This step was repeated and the blank DNA Mini Column was centrifuged at the maximum speed for 2 minutes to dry the column. After that, the column was removed from the collection tube and inserted into a new 1.5 ml microcentrifuge tube. The 100 µl elution buffer was added to the column inserted in the microcentrifuge tube, previously allowed to stand at room temperature for 2

minutes and centrifuged at the maximum speed for 1 minute. The eluted DNA was stored at -20 °C. The quality of extracted DNA was checked by polymerase chain reaction (PCR) by amplifying of the *GAPDH* gene (Bumrungthai et al., 2015) to give 117 bp of PCR product.

3.3 HPV DNA detection by real-time PCR

HPV DNA in genomic DNA extracted from FFPE ovarian samples was detected by real-time PCR using GP5+/GP6+ primers (Weynand et al., 2010) targeting to the conserved region in the L1 open reading frame of the HPV genome with an amplicon size of 150 bp. A total reaction mixture volume was 10 µl containing 2 µl extracted DNA, 5.0 µl of SsoAdvanced™ SYBR® Green Supermix, 0.2 µl of each primer at 10 µM and sterile water to reach the final volume. HPV 16 plasmid was used as a positive control. The cycling condition included initial denaturation at 98 °C for 2 minutes, 45 cycles of denaturation at 98 °C for 15 seconds, annealing at 45 °C for 30 seconds, extension at 72 °C for 30 seconds, and the final extension at 72 °C for 5 minutes. Real-time PCR product was electrophoresed through a 1.5% agarose gel at 100 volts for 35 minutes, stained with 0.5 mg/ml ethidium bromide of 15 minutes and destained with water of 15 minutes on gentle shaking. The amplified HPV DNA fragments were visualized by a UV Transilluminator.

3.4 HPV DNA genotyping by reverse line blot hybridization (RLBH)

The HPV DNA positive ovarian samples were used for HPV DNA genotyping performed as followed: the membrane (Biodyne C; Pall Corp., East Hills, NY, USA) was activated in freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide referred to as EDAC; the activated membrane was placed in a miniblotter system (Immunetics, Boston, MA, USA). Type-specific oligonucleotide probes (37 types of HR HPV) containing a 50 amino group (1st Base, Singapore) were fixed in parallel lines on the membrane; and to this was added each biotinylated PCR product that was prepared by dilution in 2X SSPE/0.1% SDS.

HPV positive samples and positive control (20 ng/µl DNA plasmid of HPV16, 33, 39, 52, 58, and extracted DNA from SiHa and HeLa cell lines) amplified with GP5+/biotinylated GP6+ primer. The 20 µl PCR product was diluted in 2x SSPE/0.1% SDS solution with a total 150 µl volume. Diluted PCR product was heated for DNA denaturation at 99 °C, for 10 minutes and cooled on ice immediately. The membrane was placed on a perpendicular pattern. The slots were filled with the diluted PCR product. Hybridization was performed by incubation at 42 °C, for 1 hour on a horizontal surface without rocking or shaking. Samples were aspirated off from the miniblotter and the membrane was removed from the miniblotter. The membrane was washed 2 times in 2X SSPE/0.5% SDS at 52 °C for 10 minutes. The membrane was incubated at 42 °C for 1 hour with 25 ml of 1:2000 diluted HRP-Conjugated Streptavidin (2X SSPE/0.5% SDS) and rotated every 15 minutes until 90 minutes. The membrane was washed three times in 1X PBS/0.15% Tween 20 at 45 °C for 10 minutes. The membrane was incubated for 3 min in a chemiluminescence solution in the dark. The membrane visualized the black spots of HPV positive signal of various HPV types by using the Amersham™ Imager 600.

3.5 Statistical analysis

The chi-square test was used to analyze the correlation between HPV infection and various histologic subtypes of ovarian cancer. A p-value < 0.05 was considered significant. All of the statistic tests were analyzed by SPSS version 17.

4. Results

4.1 The prevalence of HPV infection in the normal, benign, and EOC cases

A total of 215 FFPE ovarian tissue samples including 92 normal, 57 benign and 66 EOC cases were investigated for HPV infection. The prevalence of HPV infection in the normal, benign, and EOC cases were 25% (23/92), 26% (15/57) and 32% (21/66), respectively, as shown in Figure 1. Although the prevalence of HPV infection was not significantly different among these three groups, increasing HPV prevalence in EOC cases which was higher than others imply that HPV infection might be associated with EOC development (Figure 1). More samples are needed to further investigated.

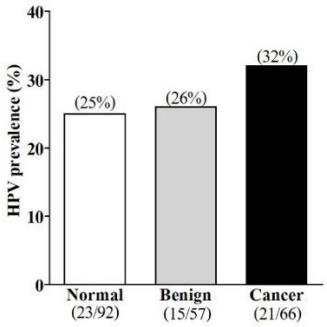

Figure 1 Prevalence of HPV in normal, benign and EOC cases

4.2 The distribution of HPV types and patterns of HPV infections (single, mixed (double/multiple) infections) in HPV-positive cases.

Figure 2 demonstrates the distribution of HPV type as follows: five HPV types in normal (HPV 6, 11, 16, 18 and 39), four HPV types in benign (HPV 11, 16, 18 and 58), and five HPV types in cancer (HPV 6, 11, 16, 18 and 39). A total of six different HPV types including HR-HPV 16, 18, 39 and 58 and LR-HPV types 6 and 11 were detected. HPV16 was the most common type followed by HPV 18 and HPV 11, displaying no significant difference was found between all ovarian groups (Figure 3).

Figure 4 shows patterns of HPV infection in all cases. Mixed (double) infection was the most common pattern and the infection of HPV 16 and 18 was the most common pattern. The HPV type infection pattern was detected with no significant difference between each group. However, mixed (double) HPV infection pattern was found higher than single in benign and cancer groups but no significant difference (Figure 5).

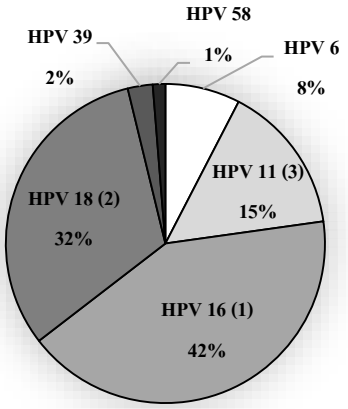

Figure 2 Distribution of HPV types in HPV-positive cases

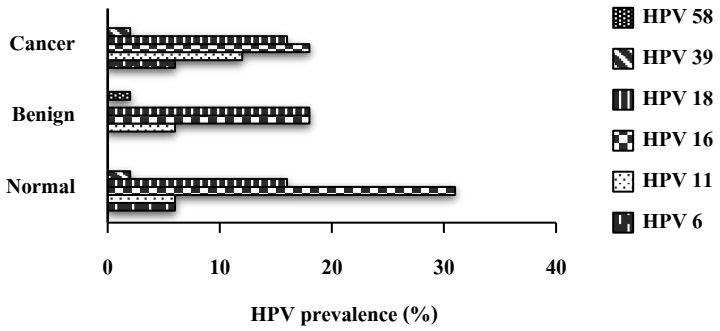

Figure 3 Group distribution of HPV types in HPV-positive cases

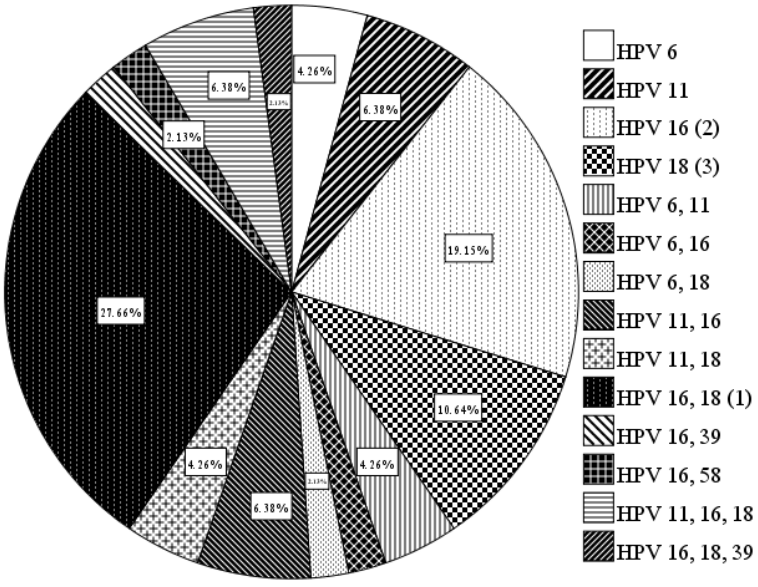

Figure 4 Percentage of HPV types in ovarian tissues

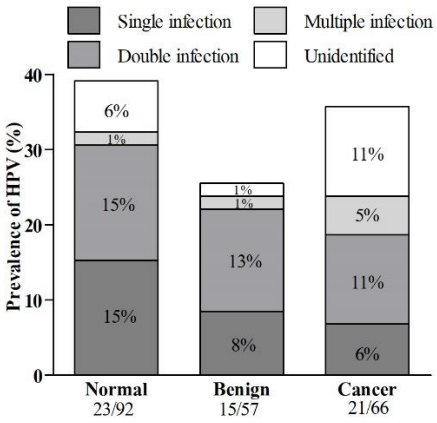

Figure 5 Group distribution of HPV infection patterns in HPV-positive cases

4.3 The prevalence of HPV in histological categories of benign epithelial ovarian tumors (serous, mucinous, borderline serous and borderline mucinous) and EOCs (serous, mucinous, endometrioid, clear cell and adenocarcinoma).

The prevalence of HPV in benign epithelial ovarian tumors was significantly higher in mucinous (11%) and serous histological subtype (12%) (Figure 6). In EOC, the prevalence of HPV in serous histological subtype (21%) was higher than other subtypes (Figure 7), however, it was not significantly different.

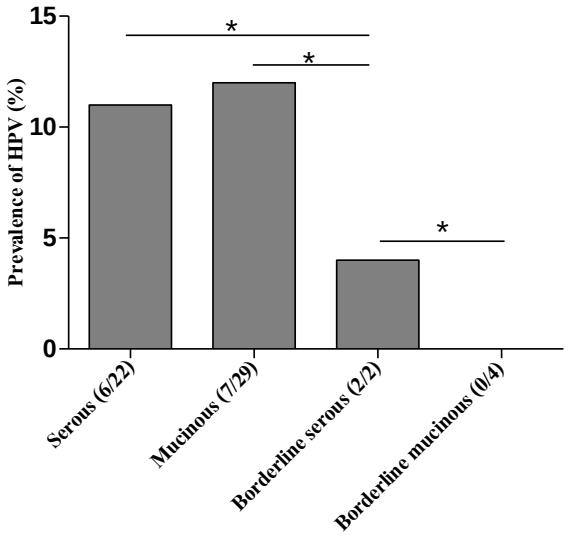

Figure 6 Prevalence of HPV in each histological subtype of benign epithelial ovarian tumors

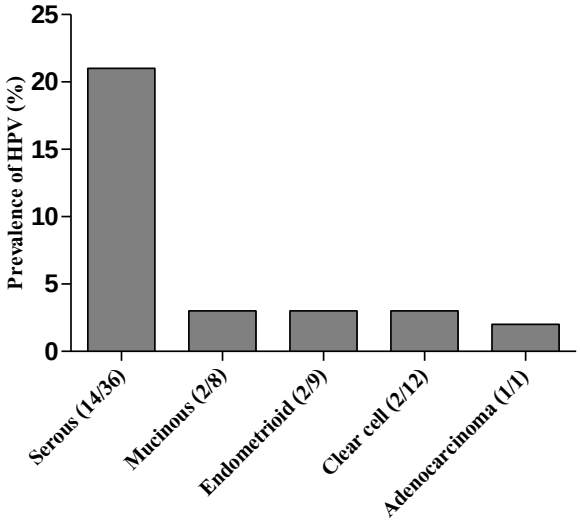

Figure 7 Prevalence of HPV in each histological subtype of epithelial ovarian cancers (EOCs)

5. Discussion

This study detected the prevalence of HPV infection and type distribution in FFPE ovarian tissues including normal, benign and EOC in northeastern Thailand. The prevalence of HPV infection in EOC in this study was 32% (21/66) lower than its counterpart found in two previous studies (37.5%) (15/40) (Kuscu, Ozdemir, Erkanli, & Haberal, 2005) and 66.7% (26/39) (Li et al., 2002). However, the prevalence of HPV infection in ovarian cancer tissue had an extensively variance of 0–66.7% (Rosa et al., 2013). The differences might be dependent on the following reasons: techniques used, the tissue samples and especially, difference in geographical regions which may be described by genetic environment or different lifestyle (Svahn et al., 2014).

The HR-HPV type 16 was the most common type detected in ovarian cancer, and the second common was HR-HPV type 18. That this study found that EOC developement might associate with HPV infections corresponds to a previous study by Atalay et al. (2007). HPV 11 was the third common in ovarian cancer. HPV type 11 are considered posing little risk for malignant transformation. However, they have been demonstrated to be associated with some progressions of invasive tumor (Byrne, Tsao, Fraser, & Howley, 1987; Gissmann et al., 1983; Manias, Ostrow, McGlennen, Estensen, & Faras, 1989). The prevalence of HR-HPV types was higher than that of LR-HPV types in all ovarian cases. Multiple infections of HR-HPV and LR-HPV were similarly frequent in all group, whereas normal group was frequently infected by HPV with more single and double patterns than in benign epithelial ovarian tumors and EOC (Figure 5). A previous study by A.-J. Chiang, Chen, Cheng, & Chang (2015) reported that multiple HR-HPV were detected in ovarian tissues. Another essential characteristic was the majority of HPV-positive cases were found in EOC, serous subtype (62.5%), the finding of when compared with other histological subtypes that was

corresponded with a report of Wu et al. who found HPV-positive cases with serous cystadenocarcinoma (62%) (Wu et al., 2003). There are many notions of entry of HPV to the ovaries. Recently, there is a hypothesis that the ovarian tumors may grow in the fallopian tube and relate to the ovary secondarily. According to what is previously mentioned, parts of dysplasia within the tubal epithelium, called "serous tubal intraepithelial carcinoma" are cleaved onto the surface covered with ovary (Erickson, Conner, & Landen, 2013). However, in this study, HPV infection was also found higher in benign serous and mucinous subtypes when compared with other histological subtypes.

6. Conclusion

HPV infections are associated with benign epithelial ovarian tumors, especially mucinous and serous histological subtype and serous subtype of EOC. Therefore, HPV infection might play a role in some specific subtypes of ovarian tumor and cancer in northeastern Thailand.

7. Acknowledgements

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