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中文題目：就人類乳突病毒型別、病毒量和其嵌入宿主細胞狀態探討臺灣婦女子宮頸癌之致癌機轉

英文題目：The Impact of Subtype Distribution, Viral Loads Disparity and Physical Status of Human Papillomavirus on the Carcinogenesis of Cervical Cancer in Taiwanese Women

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Abbreviations used

AGUS	atypical glandular cells of undetermined significance
ASCUS	atypical squamous cells of undetermined significance
CI	confidence interval
CIN	cervical intraepithelial neoplasias
CIS	carcinoma *in situ*
CT	computed tomography
FIGO	International Federation of Gynecology and Obstetrics
FISH	fluorescence *in situ* hybridization
HC2	hybrid capture 2
HPV	human papillomavirus
HR HPV	high-risk types of human papillomavirus
HSILS	high-grade squamous intraepithelial lesions
IRB	Institutional Review Board
ISH	*in situ* hybridization
LCR	long control region
LSILS	low-grade squamous intraepithelial lesions
LVSI	lymphovascular space involvement
OR	odds ratios
ORF	open reading frame
PCR	polymerase chain reaction
ROC	receiver operating characteristic (area under)
SCC	squamous cell carcinoma
SCC-Ag	squamous cell carcinoma-antigen
TCOG	Taiwan Cooperative Oncology Group

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中文摘要

累積的研究證據顯示，在子宮頸癌病患的血液內可發現腫瘤去氧核醣核酸，而血液內的腫瘤去氧核醣核酸具有診斷和臨床預後的價值。人類乳突病毒去氧核醣核酸，在子宮頸癌病患的血清和血液中，被偵測到的比率是百分之七至四十五， 而這差異可能是由於偵測樣品的不同，例如血清或血漿、萃取去氧核醣核酸的方法不同、或是使用分析的工具不同，例如傳統的聚合酶反應 (PCR)，即時定量 PCR、或是利用酵素免疫的 PCR 和不同的引子 (primers)，例如 L1、E6、E7，因此在過去的文獻報告中，只有少數是有關在血液中偵測 HPV DNA 的比較。

因為即時定量 PCR 可用來偵測在血液中 HPV DNA 的低病毒量，所以本論文的第一部採用即時定量 PCR 來改善病毒的偵測率，提供一前瞻性的人類乳突病毒偵測方法，使病人在被診斷出疾病的同一時間偵測其 HPV DNA，並討論其臨床意義。研究的結果顯示：以即時定量 PCR 的方法可以在超過四分之一(27%)的子宮頸癌病患血液中偵測到 HPV DNA。且在帶有 HPV 16, 18 或是 52 型的子宮頸癌病患中，幾乎有百分之五十的病患，可在血液裡偵測到 HPV DNA。本研究提供了子宮頸癌病患在治療後，其血液中 HPV DNA 病毒量連續追蹤的數據並強調其臨床意義的重要性。在本研究中，有 6 位子宮頸癌病患於治療完成後，其血液中偵測不到 HPV DNA，且在後續的追蹤中都沒有復發。而在連續追蹤中，有 10 位子宮頸癌病患在治療完後三個月，仍可在血液裡偵測到 HPV DNA，其中有 8 位發生轉移，且有 7 位發生的是遠端轉移。雖然本研究局限於不多的病人數，及追蹤時間不夠長，但經統計分析結果顯示：在血液裡被偵測出定量的 HPV DNA 的確反應了腫瘤轉移與否，且具有其預後價值。換言之、子宮頸癌病患在治療完後仍可在其血液中偵測到 HPV DNA，是可用來預測該病患未來復發與否的一個有用指標，並可用以決定哪一些子宮頸癌病患需要更積極的治療。

HPV DNA 嵌入宿主細胞基因體，被認為是子宮頸癌癌化轉變的重要事件，而且發生在早期子宮頸癌的階段，然而大部分的研究報告都著重在 16 型 HPV 病毒，少數著重在 18 型上。在美國、歐洲、非洲、及東南亞洲，52 型和 58 型發生在子宮頸癌病患相對是較低的，而在台灣和亞洲地區，52 型和 58 型是較常見的致癌型別。

為瞭解 HPV 52 型及 58 型，是否和 16 型及 18 型一樣其嵌入宿主細胞基因體，或是高病毒量是癌化過程中必要的，本論文第二部份的研究著重於 HPV 52 型和 58 型嵌入宿主細胞基因體的致癌過程。分析子宮頸分泌物採自 178 位連續病患、包含 81 位子宮頸癌和 97 位子宮頸中度至重度細胞病變之患者，利用基因晶片和定量即時 PCR 技術，檢查並判定 HPV16、18、52、58 型的盛行率，和其嵌入狀態和病毒量。

研究結果發現，於台灣婦女子宮頸癌病患中並不常見到 HPV 52 型和 58 型病毒 DNA 嵌入到人類基因體中，可見病毒 52 型和 58 型其 DNA 嵌入宿主基因體並非是子宮頸癌發展必須的因素。相反的，16 型及 18 型病毒 DNA 嵌入宿主基因體是導致子宮頸癌非常重要的步驟。而 16、18、52 型的高 E6 病毒量，則具有預測高度細胞病變轉變至子宮頸癌的能力。可見 HPV DNA 是否嵌入宿主基因體和其病毒量，在子宮頸癌癌化過程中，可能因病毒型別的差異而扮演不同的角色。在此研究中我們成功地利用 HPV 16、18、52 型的病毒量(the median log of viral loads) 來預測子宮頸癌，選擇的分界點可以達到預測子宮頸癌 62.5% ~83.3% 的敏感度，及 0-25%的偽陽性機率。ROC 曲線分析顯示所建立的模型能準確地預測、鑑別及診斷出高度細胞病變，或是子宮頸癌發生在病人感染 HPV 16 型、18 型或 52 型分別達 73.8%，92.9%，88.57%的正確率。

最後本論文的研究著重在低度細胞病變與病毒的相關性。幾乎 50%的非典型鱗狀上皮細胞和 80%的低度細胞病變，會被致癌型的 HPV 感染；而 HPV DNA 的測試對非典型鱗狀上皮細胞患者，可提供訊息轉介這些病患做陰道鏡檢查並查出潛藏的高度細胞病變和子宮頸癌。相反的，致癌型的 HPV DNA 檢測並無法提供低度細胞病變更一步的訊息供臨床做進一步的選擇性處理；因為在低度細胞病變患者身上有相當高的比率可偵測到 HPV DNA。而臨床上，對於低度細胞病變的處理是在 3 至 6 個月後重複做抹片檢查，或是直接陰道鏡切片檢查，所以發展出一個可供低度細胞病變患者臨床處理選擇的模式，以區分哪些患者可能會進展到高度細胞病變，哪些會自動痊癒，就非常有價值。

本研究測試在低度及高度細胞病變中，於亞洲最常見的癌前病變致癌病毒型別 HPV 16、18、52、58 四型之病毒量，並評估 2 年累積進展到高度細胞病變的危險性，

且探討病毒 DNA 嵌入宿主基因體是否為導致低度細胞病變進展到高度細胞病變的主要原因。此外，並探討在 6 個月後重複做抹片檢查時，E6 病毒量的改變是否會導致進展成高度細胞病變。研究結果發現低度細胞病變患者在 6 個月後，病毒量增加者比病毒量沒有增加的患者有 45%的危險性會進展成高度細胞病變。利用定量即時 PCR 偵測病毒量，發現病毒量增加者比沒有增加的患者有大於 7 倍的危險性會進展成高度細胞病變；若用 HC2 技術偵測病毒量發現病毒量增加者比病毒量沒有增加的患者有大於 6 倍的危險性會進展成高度細胞病變。這兩種技術偵測到的病毒量是一致的 (Person's coefficient, $r=0.687$, $p<0.001$)。結果亦指出，6 個月後重複檢測的 HPV DNA 病毒量，病毒量的改變可用來預測感染 HPV DNA 16、18、52、58 型的低度細胞病變患者，是否進展成高度細胞病變且與臨床結果相符。

總結，本論文的研究對 HPV DNA 病毒量和嵌入宿主基因體的狀態，在子宮頸癌癌化過程中所扮演的角色，提出其指標性的臨床運用，以預測癌前病變及子宮頸癌患者的疾病進展。

Abstract

Accumulating evidence shows that tumor DNA can be found in the circulation of patients with cervical cancer. The presence of such tumor DNA in the blood may be of diagnostic and prognostic value. HPV DNA has been found in serum or plasma samples from cervical cancer patients with detection rates varying from 7% to 45%. The discrepancy may be due to different target materials (serum or plasma), method of extracting DNA, tools of analysis (conventional PCR, real-time PCR, or PCR-enzyme immunoassay), and differing primers used (L1, E6, E7). Therefore, information regarding the comparison of detection rates of HPV DNA in circulating blood is limited.

The first part of this study provides a prospective study of HPV DNA detection at a single diagnostic time point. Real-time PCR is used to detect the low viral loads of HPV DNA in blood. The results show that more than one-fourth (27%) of patients with invasive cervical cancer had HPV DNA detected in their blood samples. Approximately 50% of patients with confirmed HPV 16, 18 or 52 positive cervical cancers had HPV DNA detected in their blood. This study also used serial follow-up data on HPV DNA viral load among cervical cancer patients after treatment to understand its clinical significance. Six cervical cancer patients with HPV DNA viral loads undetectable in their blood after treatment showed no recurrence during follow-up. In longitudinal follow-up, eight out of ten cervical cancer patients with viral loads of HPV DNA detectable in the blood at 3 months after treatment were associated with recurrence. Among these, seven of eight patients had distant metastases. Although the study was limited to a small number of patients and a short period of follow-up, it is worth pointing out that detection of circulating HPV DNA after treatment could predict recurrence. It is postulated that blood HPV DNA might be a useful marker to select subsets of patients who need more aggressive treatment. The presence and quantity of HPV DNA in blood are likely to be a reflection of metastasis and may be of prognostic value.

The second part of this study focuses on the role of integration of HPV type 52 and 58 in cervical cancer patients. The integration of HPV DNA into the host genome is thought to occur early in cancer development and to be an important event in malignant transformation of cervical cancer. However, most studies on the integration of HPV DNA focus on type 16 and a few on type 18. While HPV type 52 and 58 are oncogenic types

with relatively low prevalence in cervical cancer in the Americas, Europe, Africa and Southeast Asia, they are as prevalent as the known high-risk (for cervical cancer) HPV types 16 and 18 in Taiwan and other Asian countries. To analyze whether integration or high viral loads of human papillomavirus (HPV) are essential for malignant transformation of HPV type 52 and 58 as well as type 16 and 18, cervical swabs from 178 consecutive patients, including 81 with invasive cervical cancers and 97 with cervical intraepithelial neoplasias (CIN) II-III, were collected and examined to determine the prevalence, physical status and viral load of HPV type 16, 18, 52 and 58 DNA using genechip and real-time PCR (polymerase chain reaction) analysis.

The infrequent integration of HPV 52 and 58 DNA in cervical cancer suggests that it is not a prerequisite for progression to cervical cancer. By contrast, integration appears to be a critical step for carcinogenesis of HPV 16 and 18 DNA. High viral loads (E6) of HPV 16, 18 and 52 DNA may be predictive of the transition of CIN II-III to cervical cancer. The results indicate that both viral DNA physical status and viral loads of HPV are important factors in the carcinogenesis of different HPV types. This study successfully used the median log of viral loads of HPV 16, 18 and 52 DNA to predict the presence of cervical cancer. The selected cut-off values of the median log of viral loads in HPV 16, 18 and 52 DNA achieved 62.5-83.3% sensitivity and a 0-25% false positive rate in predicting the presence of cervical cancer. The ROC curve analyses indicated that the model could accurately predict the diagnostic group of CIN II-III or cervical cancer in 73.8%, 92.9%, and 88.5% of patients with positive HPV 16, 18 and 52, respectively.

The third part of this study focuses on low-grade squamous intraepithelial lesions (LSILs). Approximately 50% of atypical squamous cells of undetermined significance (ASCUS) and 80% of LSILs are infected by oncogenic types of HPV. HPV DNA testing for patients with ASCUS provides useful information and allows referral of patients for immediate colposcopy to detect high-grade squamous intraepithelial lesions (HSILs) and cancer. By contrast, oncogenic HPV DNA testing is not informative for triage of patients with LSILs because a high percentage of LSIL patients are HPV positive. A repeat Pap smear in 3 to 6 months or direct biopsy under colposcopy is generally used in clinical practice. Development of alternative triage strategies for women with LSILs would be valuable in distinguishing women with LSILs that have high probabilities of progression to HSILs from women with LSILs that have spontaneously regressed.

The 2-year cumulative risks were evaluated for HSIL attributable to HPV 16, 18, 52, and 58, the most common oncogenic types in pre-invasive cervical lesions including LSILs and HSILs in Asia, and questioned as to whether the integration of HPV oncogenes into the host genome contributed to the risk of LSILs progressing to HSILs. In addition, it was determined whether or not E6 viral load and its change contributed to the risk of LSILs progressing to HSILs during the interval between baseline diagnosis of LSIL by Pap smear and a 6-month follow-up visit by repeat Pap smear. It was found that women with LSILs whose viral loads increased between baseline and 6 month follow-up had a 45% risk of developing HSIL, which was seven-fold greater than those without increased viral loads (OR = 7.6, 95% CI = 1.9 to 29.4, $p < 0.01$), as evaluated by real-time PCR. The risk was calculated at 44%, a six-fold greater risk than those without increased viral loads (OR = 6.1, 95% CI = 1.6 to 22.7, $p < 0.01$), as evaluated by HC2. The two viral load measures correlated well (Person's coefficient, r = 0.687, $p < 0.001$). The results indicate that evaluation of viral load changes (increased or not increased) through repeat HPV DNA testing could predict progression of disease in LSIL cases of HPV types 16, 18, 52, and 58, which correlates to clinical implications.

In summary, this research strives to understand the role of HPV DNA viral loads and integration in the carcinogenesis of cervical cancer by searching for a useful marker applicable in clinical practice to predict disease progression in pre-invasive and invasive cervical cancer.

Chapter 1: General Introduction

Epidemiology of cervical cancer

Cervical cancer is a major health burden in women, with 493,000 women around the world diagnosed with cervical cancer and 273,000 dying from this cancer in the year 2002. Worldwide, cervical cancer is the second most common female cancer in the world, with a mean age standardized incidence rate of up to 18.8 per 100,000 women, and is a major cause of mortality for women in developing countries (Bosch et al, 2003). Of these, more than 80% of deaths occurred in the low and medium-resource countries of South and South-East Asia, sub-Saharan Africa, and South and Central America. In fact, one-third of the cervical cancer burden in the world is experienced in South Asia. It is also a serious health problem in Taiwan, with 2,061 new diagnosed cases and 874 mortality cases of cervical cancer in 2003, second in incidence only to breast cancer, as reported by the Bureau of Health Promotion, Department of National Health. The annual incidence of invasive cervical cancer in Taiwan was 17.2 per 100,000 (National Cancer Report, 2003).

Based on strong epidemiological evidence and supported by basic experimental findings, there is no doubt that persistent infections with high-risk types of human papillomavirus (HR HPV) represent a necessary cause of cervical cancer (Walboomers et al, 1999; zur Hausen, 2002; Bosch et al, 2003; Munoz et al, 2003). High risk HPV DNA can be detected in up to 99.7% of cervical squamous cell carcinomas (SCC) (Walboomers et al, 1999; Munoz et al, 2003) and in 94-100% of cervical adenocarcinomas and adenosquamous carcinomas (Zielinski et al, 2003). The etiological role of most common high-risk HPV types, such as types 16 and 18 in pre-invasive and invasive cervical cancers has been demonstrated by epidemiological evidence and molecular studies (IARC, 1995; Walboomers et al, 1999).

Cytology screening has been largely responsible for the significant decline in the burden of cervical cancer in developed countries over the last 5 decades. However, only around 50% annual screening rates have been achieved in Taiwan, in comparison with around 80% annual screening rates in Europe and America. Also, although the cytological screening system itself is improving, it has limited sensitivity and specificity for cervical cancer and up to 30% false-negative results still occur (Solomon et al, 2002). Women are

affected by the discomfort, complications and anxiety of the screening procedures, positive Pap smear results and follow-up investigations or treatments (Renshaw et al, 2004). In addition, adenocarcinomas develop deeper in cervical tissues than the surface cells taken for Pap smears and cervical adenocarcinoma may not initially be detected, leading to late-stage diagnosis (Schiller and Davies, 2004). With regard to prevention research, epidemiologists are currently evaluating the possibility of reinforcing screening programs by adding HPV tests to the cytology.

Age at first intercourse, number of sexual partners, high parity, cigarette smoking, race, and low socioeconomic status has consistently been shown as significant risk factors for cervical cancer. However, all these factors are linked to sexual behavior and acquisition of HPV; none have been shown to be significant independent risk factors. Sexual behavior has been consistently identified as a major risk factor for cervical cancer and population-based studies have demonstrated that risk related to sexual activity is mediated by HPV infection (Ley, et al, 1991; Bauer et al, 1993; Hildesheim et al, 1993; Silins et al, 2000; Lazcano-Ponce et al, 2001).

HPV infections are among the most common sexually transmitted infections in most populations, and estimates of exposure range from 15-20% in many European countries, to 70% in the US, 95% in high-risk populations in Africa, and 10-15% in general populations in Taiwan (unpublished data). Based on global statistics, life-time risk according to the US center for disease control for sexually active men and women is at least 50 percent (Centers for Disease Control and Prevention, 2004). By age 50, at least 80 percent of women will have acquired HPV infections. HPV infections are highly prevalent in young individuals, whereas invasive cervical cancer does not typically develop until the third decade of life or later. The overwhelming majority of women today with a diagnosis of cervical cancer have either not had regular Pap tests or they have not been followed up after detection of an abnormal smear. Not undergoing regular Pap tests is the single greatest risk factor for a poor outcome in women who develop cervical cancer. Choice of contraceptive methods also appears to affect the risk of acquiring cervical cancer and barrier mechanisms have been associated with reduced risk, whereas the use of oral contraceptives has been associated with an increased risk.

Molecular mechanisms of cervical cancer

Originally, it was thought that cervical SCC would always evolve from infected normal cervical epithelium via a continuum of long-lasting, consecutive CIN I, CIN II, and CIN III lesions. However, an alternative concept that finds increasing support is that many of the clinically relevant CIN II-III lesions may be rapidly induced within 2-3 years following infection (Winer et al, 2005), whereas it can be deduced from Wallin et al (1999) and Zielinski et al (2001) that it takes another 10-12 years to develop invasive cervical cancer.

As a consequence, most CIN I lesions and some CIN II lesions should not be considered as true precursor stages of cervical cancer, but rather the cytopathological effect of a productive viral infection. Support for this comes from studies showing that low-grade CIN lesions harbor low-risk HPV types that confer a negligible risk of progression (Lungu et al, 1992). Moreover, CIN I and some CIN II lesions that harbor HR HPV types display viral expression patterns suggestive of productive viral infections (Stoler et al, 1992; Dürst et al, 1992). In these infections, active viral replication and virion production are strongly coupled to the differentiation program of the infected epithelium. Low levels of viral activity in the infected basal cells characterize this process, with monomeric episomes being co-replicated with the genome of the host cell. Upon differentiation, viral transcription, including that of the viral E6 and E7 oncogenes, is markedly increased while vegetative DNA amplification and assembly of new virions occurs only in squamous epithelia undergoing terminal differentiation.

By contrast, some CIN II lesions, and CIN III lesions, exhibit a dramatic topographical change in viral gene expression, which includes an increase in E6/E7 expression in proliferating dysplastic cells (Stoler et al, 1992; Dürst et al, 1992). Although the mechanism underlying deregulated E6/E7 expression in proliferating cells is not yet understood, *in vitro* studies using epithelial raft cultures have shown that altered histone deacetylation may be a contributing factor (Zhao et al, 1999). In addition, high-grade CIN lesions and cervical carcinomas often show integration of the viral genome into that of the host cell (Klaes et al, 1999), a phenomenon that is accompanied by DNA aneuploidy (Melsheimer et al, 2004). In fact, integration may also interfere with the normal regulation of E6/E7 expression, either by interruption of transcriptional control mediated by the viral E2 protein, or by increased stability of chimeric E6/E7 host cell transcripts, or a

combination (Jeon and Lambert, 1995; zur Hausen et al, 2002). Although chromosomal instability is tightly linked to HPV 16 integration in cervical keratinocytes, it is still unclear whether integration represents the cause or simply the consequence of genomic instability (Pett et al, 2004).

The molecular basis for oncogenesis in cervical cancer can be understood by the regulation and function of the two viral oncogenes, E6 and E7 (Mantovani and Banks, 2001; Munger et al, 2001). These two genes have been shown to posses transforming ability when transfected into epithelial cells (Dürst et al, 1987; Münger et al, 1989). Furthermore, the persistent expression of E6 and E7 is necessary for the maintenance of the malignant phenotype (Chen et al, 1993; Seagon and Durst, 1994; von Knebe Doeberitz et al, 1994). The E6 and E7 genes are under the regulation of the E2 gene product (Bernard et al, 1989; Dowhanick et al, 1995). The integration of the viral circular DNA into the patient's genome is necessary for the malignant phenotype (Vernon et al, 1997). The E2 gene is often the site for integration resulting in disruption of the E2 gene and subsequent transcription of the E6 and E7. Viral DNA integration into host cell DNA usually disrupts the E1 and E2 open reading frames (ORFs). By contrast, the E6 and E7 ORFs and LCR (long control region), generally remain intact (Daniel et al, 1997; Lazo, 1997; Romanzuk and Howley, 1992; Syrjänen and Syrjänen, 2000). Increasing the expression of these oncoproteins (E6 and E7) was through three steps: (1), increase copy number of HPV DNA (Steenbergen et al, 1996); (2), mutations in the long control region (at YY1 motits) (Dong et al, 1994; Park et al, 1999); and (3), integration of HPV DNA into the cellular genome (zur Hausen et al, 2002; Jeon et al, 1995). Elimination of HPV 16 E2 protein expression due to integration results in up-regulation of the transcription of the E6 and E7 oncogenes possibly provides a selective growth advantage for the infected cell (Jeon et al, 1995; Jeon and Lambert, 1995).

The E6 gene product binds to the resident p53 tumor suppressor gene and induces an ubiquitin-mediated proteolysis. E7 targets another tumor suppressor, the Rb gene product. By altering its phosphorylation state, E7 functionally inactivates this protein, which, like p53, functions in cell cycle control (Dyson et al, 1989; Munger and Howley, 2002). The interactions that are thought to be most relevant for their transforming functions are E6 binding, via the cellular protein E6-AP, to the tumor suppressor gene product p53, and E7, binding to the retinoblastoma tumor suppressor gene product pRb and its related pocket

proteins, p107 and p130 (Davies et al, 1993). In addition, HR HPV E6 can activate the telomere-lengthening enzyme telomerase independent of p53 binding, and E7 can induce abnormal centrosome duplication through a mechanism independent of inactivation of pRb and its family members (Munger et al, 2004). It is likely that these latter properties also contribute to the transforming characteristics of these viral oncoproteins.

Cervical cancer is a multistep process initiated by a high-risk HPV (HR HPV) infection. Productive HPV infections are characterized by expression of the viral oncogenes E6/E7 in the differentiated cell layers, deregulated expression of E6/E7 in the dividing basal cells is suggested to represent the first step in the multistep process of HPV-mediated transformation (Munger and Howley, 2002). Interference of the viral oncogenes E6 and E7 with the apoptosis and cell cycle regulation p53 and pRb in the proliferating cells results in the induction of genomic instability (Dyson et al, 1989; Duensing et al, 2000). The genomically unstable environment provides the driving force for the acquisition of crucial alterations in oncogenes and tumor suppressor genes that are additive requirements for malignant transformation (Snijders et al, 2006).

In vitro studies show HPV could make cell lines immortalized but non-tumorigenic (Choo et al, 1994; Yokoyama et al, 1995). Expression of the high risk HPVs may lead to aneuploidy (change of chromosome number) and contribute to the other cellular events necessary for full cancer development. Analysis of the transformation of epithelial cells *in vitro* has revealed at least four consecutive stages characterized by different phenotypes, i.e., extended lifespan, immortalization, anchorage-independent growth, and tumourigenicity in nude mice (Chen et al, 1993).

Loss of tumor suppressor gene(s) at chromosome 11 has been implicated in the progression from an immortal to a tumourigenic phenotype, since introduction of chromosome 11 into SiHa cells abrogated their capacity to form tumors in nude mice, without affecting immortality (Koi et al, 1989). The significance of this finding was supported by allelotyping studies, showing frequent deletions at 11q loci, particularly 11q22-23, in cervical carcinomas (Hampton et al, 1994). Steenbergen et al (2004), collected functional evidence that the tumor suppressor in lung cancer 1 (*TSLC1*) gene (also named *IGSF4* or *NECL-2*) may be the candidate suppressor of tumourigenicity on chromosome 11. *TSLC1* gene was found to be silenced in 91% (10/11) of cervical cancer

cell lines, mostly as a result of promoter hypermethylation alone or combined with allelic loss (Steenbergen et al, 2004). *TSLC1* promoter hypermethylation was also detected in 58% of cervical carcinomas and 35% of high-grade CIN lesions, but not in low-grade CIN lesions or normal cervix (Steenbergen et al, 2004). Moreover, ectopic expression of TSLC1 suppressed both tumor formation in nude mice and anchorage-independent growth of SiHa cells (Steenbergen et al, 2004). It is interesting that from a model system of non-tumorigenic hybrids of the HPV 18-containing cervical cancer cell line, HeLa with fibroblasts and their tumorigenic segregants, a change in the composition of the AP-1 complex emerged as being relevant for tumorigenicity (Soto et al, 1999; Finzer et al, 2000).

Characteristics of human papillomavirus

The HPV genome consists of eight kilobasepairs, and is a non-enveloped double-stranded DNA molecule. The genome can be divided into three regions; the long control region (LCR) without coding potential, the region of early proteins (E1-E8), and the region of late proteins (L1 and L2). E6 and E7 are the most important oncogenic proteins. Transcription of the E6 and E7 genes was observed always to occur in cervical carcinomas and this was the first indication of an important role for these genes in HPV-associated tumorigenesis. The E6 ORF encodes a small protein of approximately 150 amino acids with a molecular weight of 16-18 kD. The E6 protein of HR anogenital types shows only weak oncogenic potential in most established cell lines and cooperation with the E7 protein is required for full transforming and immortalizing capacity. The E7 ORF encodes for a small protein of approximately 100 amino acids with a molecular weight of 10 kD. E7 is the major transforming oncogene of HPVs (Burd, 2003).

HPV's belong to the family *Papovaviridae*. They consist of a 72-capsomere capsid containing the viral genome. The capsomeres are made of two structural proteins; the 57kDa late protein L1, which accounts for 80% of the viral particle, and the 43-53 kDa minor capsid protein L2. L1 is major capsid protein and has an immunogenic role. E6 and E7 are oncoproteins. E1 and E2 maintain a stable viral episome and initiate viral replication. The E1-E2 junction is the site of integration. E4 probably facilitates the release of newly formed virions from the cell. E5 results in neoplastic transformation in some experimental systems (Burd, 2003).

The evidence of HPV infection and cervical carcinogenesis is based on the E6 and E7 of high-risk HPV types can transform cells in culture (Munger and Howley, 2002). Integrated forms from HPV DNA 16 or 18 in cervical cancer was found in contrast to that of episomal forms from type 6 or 11 in condydoma (Badaracco et al, 2002; Thomas et al, 2001). The interaction of E6 of HPV 16 and 18 is from binding with p53 in contrast E7 with Rb.

The integrated state revealed E2 disruption from E1-E2 junction and caused E6, E7 expression (Daniel et al, 1997; Lazo, 1997; Romanzuk and Howley, 1992; Syrjänen and Syrjänen, 2000). When HPV DNA integrated into host cellular genome, E2 function lost could not inhibit E6 and E7 function. The key action of HR E6 proteins is the inhibition of the function of p53, a tumor suppressor protein, by enhancing its degradation through the ubiquitin pathway (Mantovani and Banks, 2001). In non-infected cells, the ubiquitin mediated degradation of p53 is triggered by the mdm-2 protein, while in HR HPV infected cells the E6 AP complex replaces mdm2 in the control of cellular p53 levels (Huibregtse et al, 1993; Scheffner et al, 1990). Most E6 proteins from LR HPVs do not bind to p53, and none of them induce its degradation (Huibregtse et al, 1993). HR HPV E6 proteins lead to a down-regulation of p53-dependent transcription, and E7 acts by binding cellular proteins of the pRB tumor suppressor family, which, by interacting with the E2F family of transcription factors, control cell replication. Binding of E7 to the active form of pRB leads to the release of the E2F transcription factors, which stimulates entry into the S-phase of the cell cycle and leads to cell replication (Munger et al, 2001).

In vitro studies show HPV can make cell lines immortalized but non-tumorigenic. Other factors are necessary for the development of cervical cancer such as environmental factors, host related factors and viral factors (Choo et al, 1994; Yokoyama et al, 1995; Tjalma, 2005). The environmental factors include smoking, parity, oral contraceptives, sexual transmitted disease and diet. The host related factors include endogenous hormones, immune response, and genetic susceptibility traits such as specific HLA alleles. The viral factors include HPV type and variant, viral load and viral integration (Choo et al, 1994; Yokoyama et al, 1995; Tjalma, 2005).

As a consequence of chromosomal instability induced by deregulated viral gene expression, viral genomes or fragments may become integrated into the host cell

chromosomes. There is no specific integration locus within the host cell genome, although fragile sites are preferred as integration sites (Wentzensen et al, 2004). In contrast, the viral genome reveals few highly characteristic features. The consequences of HPV integration include increase of messenger RNA half-life, abrogation of the E2 repression effect, position effects on viral transcription, insertion mutagenesis and target for epigenetic modification (Bhattacharjee and Sengupta, 2006; Wilke et al, 1996; Jeon et al, 1995). In all analyzed cervical carcinoma cells, a cassette consisting of the viral promoter and enhancer element located in the upstream regulatory region, and the genes E6 and E7 are retained intact on integration (Jeon et al, 1995). The cassette is concisely transcribed, and the fusion of the viral sequences with cellular sequences that are co-transcribed at the 3' end of the transcript favor the stability of these transcripts, resulting in a higher oncogenic potential (Jeon et al, 1995). Although HPV 16 and 18 are the most common HPV types in cervical cancer, a recently pooled analysis of case-control studies world-wide led to the classification of 15 different HPV types as HR HPV types, with another three types considered probably high risk (Munoz et al, 2003). HPV's were originally classified into cutaneous types such as HPV 1, 4, 10, etc, and mucosal types such as HPV 6, 16, 18, etc (Chan et al, 1992). The anogenital HPV's have been divided into two groups: the first is associated with a high risk for cervical cancer development - the HR HPVs (16, 18, 26, 31, 33, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82), and the second group with a low carcinogenic potential - the low-risk (LR) HPV's (6, 11, 40, 42, 43, 44, 54, 61, 72 and 81) (Munoz et al, 2003).

Oncogenic properties of HPV

It is well known that HR HPV infections, although necessary, are far from sufficient for the pathogenesis of cervical cancer. In fact, cervical cancer is only a rare complication of HR HPV infection and requires several additive conditions and events to accumulate once an infection has become evident. This is particularly exemplified by the fact that HPV infections are very common in young women but frequently resolve spontaneously. The life-time risk of contracting HPV is estimated to be 80% (Baseman and Koutsky, 2005), and at least 80% of the HR HPV infections are likely to be transient, not even giving rise to CIN lesions (Meijer et al, 2000).

In recent decades, the oncogenic properties of HPV have been intensively studied and significant progress has been achieved in the investigation of HPV prevention. More

than 35 types of HPV infect the genital tract, type 16 and 18 inducing about 70% of cervical cancers and high-grade of cervical intraepithelial neoplasia, and HPV type 6 and 11 causing 90% of genital warts (Muñoz et al, 2004). A prophylactic vaccine that targets these types would substantially reduce the burden of HPV-associated clinical disease. The oncogenic properties of HPV probably require specific viral types in conjunction with specific environmental and host-defined factors. A multistep pattern of cervical cancer development postulates that specific types of HPV produce an infection months to years before the development of invasive carcinoma, but only selected patients proceed to develop the carcinoma, which is preceded by a series of precursor lesions. Originally, the transforming properties of HR HPV types in epithelial cells were established by studies showing their capacity to induce immortalization of primary human keratinocytes (Dürst et al, 1987). Upon prolonged culturing, it was found that fully tumorigenic clones could emerge from HPV-immortalized cells (Chen et al, 1993; Seagon and Durst, 1994). This is in line with the assumption that HR HPV alone can induce malignant growth as long as genomic instability triggered by its E6/E7 functions is allowed to proceed uninterrupted, leading to the accumulation of relevant additive (epi) genetic changes.

The inactivation of Rb as a result of deregulated HR HPV expression is characterized by a permanent up-regulation of the cyclin dependent kinase inhibitor owing to a disruption of the Rb dependent negative feedback loop regulation $p16^{INK4A}$ expression (Lieskovska et al, 1999). In line with this, diffuse $p16^{INK4A}$ immuno-histochemical staining patterns can be found in HR HPV-containing high-grade CIN lesions, cervical carcinomas and carcinomas of other anogenital sites that are HR HPV related, such as penile carcinomas (Murphy et al, 2003).

The uncontrolled cell proliferation resulting from the inactivation of Rb and concomitant induction of E2F is also reflected in an altered expression of proliferating markers, such as minichromosome maintenance proteins (MCMs), PCNA and Ki-67 in CIN lesions and cervical carcinomas (Keating et al, 2001; Williams et al, 1998). Viruses considered related to cervical cancer are human papillomavirus, herpes simplex virus, and human immunodeficiency virus. Several HPV related cancers are reported including cervical cancer, penile cancer, vulva cancer, vaginal cancer, colorectal cancer, esophageal cancer, lung cancer, and head and neck cancer.

Biological Importance of this Study and Hypothesis

Accumulated evidence shows that tumor DNA can be found in the circulation of patients with cervical cancer (Stroun et al, 2000; Anker et al, 1999). The presence of tumor DNA in the blood may be of diagnostic and prognostic value. Genetically or even epigenetically altered tumor DNA present in the primary tumor can also be detected in the serum or plasma (Nawroz et al, 1996; Sanchez-Cespedes et al, 2000). Cervical cancer is the leading cancer among women in Taiwan. Liaw et al studied the association between HPV infection and cervical cancer in Taiwanese women and found that 10% of low-grade and 33% of high-grade cervical neoplasias could be explained by infection with HPV subtypes 52 and/or 58 (Liaw et al, 1995). Huang et al reported that HPV-52 and -58 were as prevalent as the high-risk HPV types 16 and 18 in Chinese women (Huang et al, 1997).

HPV DNA has been found in serum or plasma samples from cervical cancer patients with detection rates ranging from 7% to 45% (Capone et al, 2000; Dong et al, 2002; Widschwendter et al, 2003; Pornthanakasem et al, 2001; Liu et al, 2001). The discrepancy in these rates may be due to differences in target materials (serum or plasma), methods used to extract DNA, tools used to analyze DNA (either conventional PCR, real-time PCR, or PCR-enzyme immunoassay) and in primers selected (L1, E6, E7). Few studies have compared detection rates of HPV DNA in circulating blood (Capone et al, 2000; Dong et al, 2002; Widschwendter et al, 2003; Pornthanakasem et al, 2001; Liu et al, 2001). In addition, several studies have suggested that HPV viral load might play an important role in the progression from HPV infection to cervical cancer development (Josefsson et al, 2000; Ylitalo et al, 2000). Testing of this hypothesis via prospective study with HPV DNA detection at diagnosis and during follow-up is mandatory. Therefore, whether the detection of HPV type 16, 18 and 52 DNA in the peripheral blood of patients with cervical cancer using real-time PCR is feasible and correlates with clinical prognosis will be examined in this study.

Previous studies suggested that benign HPV lesions and CIN I lesions mostly contain the viral sequences only as episomes (Choo et al, 1987; Jeon et al, 1995). In contrast, viral DNA is integrated into the host genome in virtually all cases of cervical carcinomas and their derivate cell lines (Boshart et al, 1984; Vernon et al, 1997). Study by Peitsaro et al (2002), revealed that integrated HPV type 16 is frequently found in cervical cancer precursors, and rapid progression of the CIN lesions was closely associated with a heavy

load of integrated HPV 16. HPV 16 DNA integration has been associated with malignant transformation, with increased frequency from CIN to invasive cancers (Nagao et al, 2002). In contrast, a significant proportion of advanced cancers containing only the episomal form of HPV DNA have demonstrated that HPV16 integration might not be essential for malignant transformation (Badaracco et al, 2002; Das et al, 1992). On the other hand, the presence of HPV 18 may be associated with an aggressive phenotype which more rapidly progresses from dysplasia to invasive cancer. One very important finding is that 100% of HPV 18 positive cervical carcinomas contain integrated viral DNA only (Badaracco et al, 2002). In addition to integration, heavy viral loads in CIN lesions have recently been shown to increase the risk of carcinoma development *in situ* by at least 60-fold (Josefsson et al, 2000). However, whether viral load or integration status of HPVis a risk factor for cervical cancer progression remains unclear due to the conflicting results obtained using different methodologies in previous studies. In addition, data on the physical state of HPV 52 and 58 DNA in patients with CIN II–III and invasive cervical cancer have not been reported. This study will analyze whether integration or high viral loads of human papillomavirus (HPV) is essential for malignant transformation of HPV types 52 and 58, as well as types 16 and 18.

Approximately 50% of atypical squamous cells of undetermined significance (ASCUS) and 80% of LSILs are infected by oncogenic types of HPV (ALTS group, 2000; Solomon et al, 2001). HPV DNA testing for patients with ASCUS provides useful information and allows referral of patients for immediate colposcopy to detect high grade squamous intraepithelial lesions (HSILs) and cancer (Solomon et al, 2001). In contrast, oncogenic HPV DNA testing is not informative for triage of patients with LSILs because a high percentage of LSIL patients are HPV positive (ALTS group, 2000). A repeat Pap smear in 3–6 months or direct biopsy under colposcopy is generally used in clinical practice. Development of alternative triage strategies for women with LSILs would be valuable in distinguishing women with LSILs that have high probabilities of progression to HSILs from women with LSILs that have spontaneously regressed.

Due to the lack of sufficiently large prospective longitudinal follow-up studies and the different geographic distributions of HPV types, it has not been established how the risk of HSILs differs during transition by the physical status of HPV 16, 18, 52, and 58 DNA (integrated versus episomal DNA) during longitudinal follow-up, the viral loads

(high vs. low) and the viral load change (increased vs. not increased) between baseline and follow-up among women with LSILs. Thus it is important to have better prediction of the risk of progression of low-grade squamous intraepithelial lesions (LSILs) of the uterine cervix in women with human papillomavirus (HPV) infections.

Specific Aims

Following questions are aims to achieve in this proposal: (1) Whether the genotype and viral load of HPV in blood and cervical swabs is correlated? (2) Whether the detection of HPV type 16, 18 and 52 DNA in the peripheral blood of patients with cervical cancer using real-time PCR is feasible and its correlation of clinical prognosis. (3) Whether integration or high viral loads of HPV is essential for malignant transformation of HPV types 52 and 58 as well as types 16 and 18? (4) Whether integration or high viral loads of HPV predict prospectively the risk of progression of low-grade squamous intraepithelial lesions (LSILs) of the uterine cervix in women with HPV infections.

Chapter 2: Materials and Methods

Evaluating the feasibility of detecting HPV type 16, 18 and 52 DNA in the peripheral blood of patients with cervical cancer using real-time PCR and to determine its clinical significance:

A hospital-based study was performed to determine the prevalence of type 16, 18 and 52 HPV infections not only in cervical swabs but also in blood among women with pre-invasive and cervical cancer residing in Taipei, Taiwan. Blood and cervical swab specimens from 135 consecutive patients with 60 invasive cervical cancers, 10 microinvasions, 20 cervical intraepithelial neoplasias (CIN III, 10 CIN II, 10 CIN I) and 25 controls, were collected and examined for HPV type 16, 18 and 52 DNA using real-time PCR to investigate the prevalence and viral load of HPV DNA at the time of diagnosis and during follow-up in patients with positive blood samples.

This study was designed to provide new information regarding the occurrence of cervical cancer in patients with HPV subtype 52 detected in circulating blood, a relatively uncommon finding among cervical cancers in the Americas, Europe, Africa and Southeast Asia. The study protocol was reviewed and approved by the Institutional Review Board and Research Grant Committee of Cathay General Hospital. A total of 135 patients treated in the gynecologic cancer center of Cathay General Hospital (CGH) from January 2003 to December 2003 were recruited. Patients were recruited consecutively from those diagnosed with CIN lesions during examinations between January and June 2003. Among the 135 patients, 25 patients with benign tumors who received a simple hysterectomy performed by the same surgeon were recruited between January and March 2003 to serve as controls. The controls had no pathological findings of CIN or cancer of the cervix.

Justification of the sample size is that this study was designed to detect a clinically meaningful difference in the prevalence rates of positive test results for HPV DNA in blood samples between patients with invasive cervical cancer and patients with CIN or the controls. Assuming that the incidence rate of positive test results for HPV DNA in blood samples for patients with CIN or the controls is less than 2% (Dong et al, 2002), the selected sample size of 130 (65 per group) will give a 97% probability of correctly

detecting a 25% difference in the prevalence rate of positive test results for HPV DNA in blood samples at the 5% level of significance. Both blood specimens and cervical swabs were collected from all patients for the viral load detection of HPV types 16, 18 and 52 using real-time PCR. However, two of the 60 invasive cervical cancer specimens from cervical swabs could not be obtained due to lack of permission from patients.

All patients underwent complete physical and gynecologic examination, and the cervical cancer was staged according to the guidelines of the International Federation of Gynecology and Obstetrics (FIGO). By the end of December 31, 2004, all patients had follow-up after primary treatment at 3-month intervals for the first two years or until death. All surviving patients were followed up for at least one year. The follow-up investigations included physical examination, cervical cytology, chemistry profiling and analysis of tumor markers such as serum squamous cell carcinoma antigen (SCC-Ag) or CA-125. Further examinations, such as computed tomography (CT) of the pelvis, bone scan, chest radiography or biopsy of any suspected lesions, were performed when clinically indicated. Distant metastasis was defined as disease that occurred outside the pelvis. The association was evaluated between the HPV DNA level in the blood and clinical parameters such as tumor size, clinical staging, tumor marker, lymph node metastasis, lymphovascular space involvement (LVSI), histological type and adjuvant therapy. Among patients with a HPV-positive blood sample, the correlation between the amount of HPV DNA in blood and clinical parameters was evaluated before treatment and at three months after treatment.

DNA from cervical swab or blood samples was extracted using the QIAamp Blood Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Quantitative real-time PCR fluorescent assays for each of the HPV genotypes and for the HLA-DQα gene were performed with the Qgene HPV 16, HPV 18, HPV 52 and housekeeping gene HLADQα detector kits (Qgene Biotechnology, Kaohsiung, Taiwan) along with SYBR Green dye using the ABI 5700 apparatus (Applied Biosystems, Foster City, CA). The cervical swab was taken with an Ayre spatula and agitated into 3 ml of Tris-HCl (pH 8.3), 0.2% Triton X-100, then stored at –20°C. Two ml of whole blood was obtained and stored in a tube containing citric acid. The real-time PCR assay had a dynamic range from 10^0 to more than 10^7 copies, allowing documentation of a wide range of HPV DNA copies found in the clinical specimens.

Samples were stratified by type of cervical disease, viral type and the range of viral DNA copies per microgram of cellular DNA, also the ability of these parameters to predict the progression of cervical carcinoma was analyzed. Blood and cervical swabs from women with CIN or cervical cancer were tested for the presence of HPV type 16, 18 and 52 DNA using real-time quantitative PCR and the presence and viral load of HPV NDA in blood and cervical swabs was correlated with CIN lesion status and the different stages of cervical cancer.

Preparation of DNA from blood and cervical swabs

DNA was extracted for PCR by adding a 400-µl aliquot of a swab sample to 500 µl of DNA extraction solution (Qiagen, Hilden, Germany) with proteinase K, and then incubated at 56°C for 1 h. The Proteinase K was then heat inactivated at 100°C for 30 minutes. After centrifugation at 10,000 xg for 10 minutes at 4°C, the supernatant was collected and transferred to a new microcentrifuge tube. Next, 400 µl of the solution from the cervical swab sample was processed using the QIAamp Blood Kit (Qiagen) according to the protocol recommended by the manufacturer. For blood samples, a 400-µl aliquot lysate was incubated with the Qiagen protease and buffer AL from the QIAamp Blood Kit (Qiagen) at 56°C for 10 minutes. The lysate was applied to a QIAamp spin column, and finally eluted with 100 µl nuclease free water (QIAamp Blood DNA mini kit protocol), and vortexed for one minute before PCR amplification.

Real-time quantitative PCR of HPV type 16, 18, 52 and HLA-DQα DNA

Real-time PCR was performed with the Qgene HPV 16, HPV 18, HPV 52 and housekeeping gene HLA-DQα detector kits (Qgene Biotechnology, Kaohsiung, Taiwan) in a 15 µl reaction mixture and was monitored after each elongation step by SYBR Green 1 dye binding to amplify product using the ABI 5700 apparatus (Applied Biosystems, Foster City, CA). Highly specific primers were selected and hot start PCR was used to reduce the interference of non-specific primer annealing.

Quantitation was done using an external standard curve. The HPV type 16 viral DNA fragment (415 bp) was amplified from genome of the CaSki cell line (Baker et al, 1987), and a 415-bp long HPV 18 viral DNA fragment was amplified from the genome of

a HeLa cell line (Schwarz et al, 1985). A 50-bp long HPV type 52 viral DNA was amplified from the positive clinical samples. The accurate molecular weight and copy numbers of each viral DNA fragment were determined based on UV absorption at a wavelength of 260 nm, and then used as the template DNA for establishment of the standard curve. A standard curve with each template at 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 copy numbers was generated in parallel with the clinical samples. Concentrations of HPV DNA were expressed as copies of HPV genome per 1 μg of cellular genome from the cervical swab or copies of HPV genome in each ml of blood.

The HPV 16, HPV 18, HPV 52 and HLA-DQα primer sequences from each kit are shown in Table 1. An aliquot of 5 μl of DNA sample was added to 10 μl of PCR reagent mixture consisting of HPV optimal buffer (15 mM KCl, 20 mM Tris-HCl (pH 8.3), 0.2% Triton X 100, nuclease-free bovine serum albumin, and $MgCl_2$), which was optimized to obtain a specific and efficient amplification - 0.3 pmol/μl of each primer, 1 mM each dATP, dCTP, dTTP, dGTP, 3.75 μl 1X SYBR Green and 0.07 U/μl Ampli*Taq* Gold (Roche Molecular Systems, Foster City, CA). Following the addition of the sample, the microamp tubes were capped and then centrifuged at 1,000 xg briefly. The reaction was started with a 10 minute incubation at 95°C to activate the Ampli*Taq* Gold, followed by 50 cycles at 95°C for 30 seconds, then 60°C for 30 seconds and finally 72°C for 45 seconds. The specificity was verified on the dissociation curve as well as by electrophoresis on 2% agarose gel. A linear plot of the log of copy numbers vs. numbers of threshold cycles was consistently obtained for HPV type 16, 18 and 52 genes, and the correlation coefficient for each target gene was between 0.995 and 1.00 in each run.

Validation of Real-time PCR quantitation

Both the HPV and HLA-DQα PCR reactions were performed in duplicate. Multiple negative water blanks were included in every analysis. Standard curves were run in parallel with each analysis using DNA extracted from HPV-positive cell lines (CaSki cells were derived from HPV 16 integrated human cervical carcinoma and HeLa cells from HPV 18 integrated human cervical adenocarcinoma; both obtained from the ATCC). The PCR product of HPV DNA 52 was obtained as type 52 positive controls from a clinical sample confirmed by direct sequencing. CaSki DNA and HeLa DNA were used to further validate the accuracy of the real-time PCR. CaSki cells are known to contain about 600 copies of HPV 16 genome per cell (equivalent to 6.6 pg of DNA/genome)

(Baker et al, 1987). Because the weight of one genome per cell is approximately equal to 1.1 (6.6/600) pg, the total cellular CaSki DNA was estimated to contain about 10,000 copies of the HPV genome. The real-time PCR results revealed that at 1.1 ng of CaSki DNA input, the amplification plot overlapped with the standard at 10,000 copies of amplified DNA from genomes of the Caski cell line and also of the HeLa cells.

The normal and affected samples from the 135 patients were run in duplicate. Primers and probes to a housekeeping gene (HLA-DQα) were run in parallel to standardize the input DNA. Concentrations of blood HPV DNA were expressed as copies of HPV genome/ml of blood and were calculated using the following equation (Lo et al, 1999) C = Q x Vdna/Vpcr x 1/Vext, where C represents the target concentration in blood, expressed as copies/ml; Q represents the copy number as determined by the sequence detector; Vdna represents the total volume of DNA obtained after DNA extraction (50 μl); Vpcr represents the volume of DNA used for the PCR reaction (5 μl); and Vext represents the volume of blood used to extract the DNA.

Direct sequencing of products of the Real-time PCR

All products of the real-time PCR were purified using a pre-sequencing kit and then sequenced with the HPV type 16, 18 and 52 specific primers (Table 1) and a DNA sequencing kit. Finally, the sequencing products were purified using ethanol precipitation and were analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster, CA). Sequence alignments were obtained using returned results from the GeneBank on-line Blast server (from URL: http://www.ncbi.nlm.nih.gov/BLAST/).

Differences in proportions were evaluated using Fisher's Exact Test. A value $p<0.05$ was considered significant. Statistical analysis was performed using Statistical Package for the Social Sciences software (SPSS, Chicago, IL).

Analyzing whether integration or high viral loads of human papillomavirus (HPV) is essential for malignant transformation of HPV type 52 and 58 as well as type 16 and 18:

Sample collections

The study protocol was reviewed and approved by the Institutional Review Board

and Research Grant Committee of Cathay General Hospital (CGH). This prospective study was conducted to determine the prevalence, physical status and viral load of the HPV types 16, 18, 52 and 58 DNA in the cervical swabs of patients with CIN II-III and cervical cancer using genechips and real-time PCR analysis. Between January 2003 and March 2004, at the gynecologic cancer center of CGH, cervical swabs were collected at the time of diagnosis of cervical carcinoma from 81 consecutive Taiwanese patients and from 97 consecutive Taiwanese patients at the time of their diagnosis with CIN II-III. Informed consent for participation was obtained from each patient. The cervical swab was collected with an Ayre spatula and agitated into 3 ml of Tris-HCl (pH 8.3), 0.2% Triton X-100, then stored at -20^{o}C.

DNA Extraction

DNA was extracted with the QIAamp DNA Blood Mini Kit (Qiagen Catalog No.51106) according to the manufacturer's instructions. Extracted DNA was eluted with 100 μl AE buffer (10 mM Tris, pH8.5) and stored at -20^{o}C until analysis.

Diagnosis of disease and follow-up of patients

All patients with CIN II-III or cervical cancer were examined by colposcopy and the diagnosis was confirmed by biopsy. All patients underwent complete physical and gynecologic examination, and the cervical cancer was staged according to the guidelines of the International Federation of Gynecology and Obstetrics (FIGO). All patients were followed up after primary treatment at 3 month intervals for the first 2 years or until death. Follow ups, including physical examination, cervical cytology, blood chemistry profile, and tumor marker detection such as SCC or CA-125, and further work up such as computer tomography (CT) scans of the pelvis, bone scan, chest radiograph, or biopsy of any suspected lesions, were performed when clinically indicated. Associations were evaluated between HPV status and viral load in swabs and clinical parameters such as tumor size, clinical staging, tumor marker, lymph node metastasis, lymphovascular space involvement (LVSI), histological type, adjuvant therapy and clinical outcome.

HPV DNA genotyping

The frequency of HPV DNA and genotypes were determined by a polymerase chain reaction-based (PCR) genechip method with HPV L1 gene MY11/Gp6+ consensus primers as previously described (Huang et al, 2004). The MY11/GP6+ consensus primers

were used to amplify a fragment of 190 bp in the L1 open reading frame.

E2, E6 viral load absolute quantitation of HPV type 16, 18, 52 and 58 using real-time PCR

DNA amplifications were carried out in a 96-well plate in an ABI Prism 5700 Sequence Detection System (Applied Biosystems). Both E2 and E6 real-time polymerase chain reactions were carried out in triplicate for each sample. Amplification and quantification of the E2 and E6 genes were carried out simultaneously in separate reactions in a 96-well PCR plate. Additionally, one sibling control sample, which had been quantified previously, was included in each reaction to serve as quality assurance of the quantification system. Numbers of the threshold cycle (Ct) obtained from E2 PCR and those from E6 PCR were regressed to the standard curve to obtain the HPV copy number. For evaluation of triplicate data, the mean value and standard deviation (SD) were calculated. Data between the ranges of mean ±1.96x SD was considered acceptable. Multiple negative water blanks were included in every analysis.

The reaction was performed in a 25 μl mixture containing 1x reaction buffer (HPTM HotStart Taq SYBR Green Kit Cat No.PTM767B, Protech) and 100 nM of primers for both E2 and E6 regions. Fifty nanograms of total DNA were added to the reaction mixture. The primer sequences of E2 and E6 for HPV type 16, 18, 52 and 58 were as shown in Table 3. The amplification conditions were as follows: 10 min at 95^{o}C, a two-step cycle at 95^{o}C for 10s and 60^{o}C for one minute for a total of 45 cycles. The specificity was verified by the additional dissociation curve and followed by 2% agarose gel electrophoresis. Two standard curves were obtained by amplification of serial dilutions (ranging from 10 to 10,000,000 copies per μl) of cloned plasmid containing either partial HPV 16 (from base 28 to base 3890), HPV 18 (from base 45 to base 3993), HPV 52 (from base 95 to base 3895) or HPV 58 (from base 45 to base 3994) DNA, which included equivalent amounts of E2 and E6 genes in pGEM T-Easy vector (Promega). The number of threshold cycles for E2 PCR and E6 PCR were equivalent in each run. A linear plot of the log of the copy number vs. number of threshold cycles was consistent for both genes, and the correlation coefficient was between 0.995 and 1.00 in each run.

Assumptions of physical status using E2/E6 ratio

The real-time PCR methods used in this study were developed based on the

following assumptions: (1) preferential disruption of E2 will cause the absence of E2 gene sequences in the PCR product following integration, (2) copy numbers of both genes (E2 and E6) should be equal when viral DNA presents in episomal forms, and (3) E2 gene copy numbers will be smaller than that for E6 when viral DNA presents in concomitant form.

Measurement of E2/E6 ratio in relation to physical status

Concentrations of HPV DNA were expressed as copies of HPV genome in 50 ng of cellular DNA. Ratios of E2 to E6 less than one indicated the presence of both integrated and episomal forms. The amount of integrated E6 was calculated by subtracting the copy numbers of E2 (episomal). The ratio of E2 to integrated E6 represented the amount of the episomal form in relation to the integrated form.

Statistical analysis

Statistical analysis was mainly performed using SAS 9.1.3 software. The viral load was analyzed using a Wilcoxon rank sum test based on log transformed data ($y = \log(x+1)$) to compare differences between groups. The frequency distribution of physical status was analyzed by Fisher's exact test. A diagnostic test based on log transformation of E6 viral loads was used to perform receiver operating characteristic (ROC) curve analysis.

Analyzing whether integration or high viral loads of HPV predict prospectively the risk of progression of low-grade squamous intraepithelial lesions (LSILs) of the uterine cervix in women with human papillomavirus (HPV) infections:

To examine this issue, the 2-year cumulative risk were evaluated for HSIL attributable to HPV 16, 18, 52, and 58, the most common oncogenic types in pre-invasive cervical lesions including LSILs and HSILs in Asia, and questioned whether the integration of HPV oncogenes into a host genome contributed to the risk of LSILs progressing to HSILs. In addition, it was determined if E6 viral load and its change contributed to the risk of LSILs progressing to HSILs during the 6 month interval between baseline diagnosis of LSIL by Pap smear and the 6 month follow-up visit by repeat Pap smear.

Subjects and Methods

The Taiwan Cooperative Oncology Group (TCOG) (T1899), a multicenter study, was conducted under the supervision of the National Health Research Institute of Taiwan. A total of 1246 women with abnormal Pap smears, including those diagnosed with ASCUS or AGUS (n = 431), LSIL (n = 437), and HSIL (n = 373), from August 1999 to March 2004, were enrolled. The details of the study design and population have been published (Chen et al, 2006; Sun et al, 2005). Of the 1246 participants, 936 underwent cervical biopsies for histologic examination.

Women with LSIL (n = 437) had Pap smears, HPV testing, and colposcopic examinations every 3 months during the follow-up period. Participants were excluded from the study if they had no follow-up data (n = 60), baseline cancer on pathological examination (n = 1), fewer than 4 follow-up visits (n = 82), or no baseline HPV data (n = 2). Women with histologically confirmed HSIL were defined as having disease progression and were treated and exited from the study. Women with LSIL with two consecutive normal Pap smears and who showed HPV clearance during the follow-up period were defined as in remission and also exited. Women with LSIL not in remission or progression were defined as having persistent disease. HPV persistence was defined as HPV positivity for a given type tested on two consecutive occasions versus clearance.

A total of 294 women with LSIL, having at least four follow-up visits every 3 months, and 460 women with HSIL were tested for HPV DNA using both HC2 and PCR-reverse line blotting and were included in the longitudinal follow-up study (unpublished data). Among the 294 patients with LSIL, 187 specimens from 65 women with HPV 16 (n = 14), 18 (n = 8), 52 (n = 30) and 58 (n = 13) were collected at baseline and followed up every 6 months until follow-up showed disease progression. The specimens were further tested for viral load, E2/E6 ratio and viral load change using real-time PCR. Four who lacked samples of follow-up HPV DNA were excluded from final analysis. In addition, 212 HSIL positive patients with HPV 16 (n = 92), 18 (n = 5), 52 (n = 57), or 58 (n = 58) infections were also obtained and tested for viral load, E2/E6 ratio, and physical status of HPV DNA using real-time PCR to compare with data from LSIL specimens. Specimens for viral load and viral load change by hybrid capture two were also tested and these results compared to those obtained by real-time PCR to predict disease progression.

HPV DNA testing - HC2 and PCR methods

HC2 (Digene Corporation, Silver Spring, MD, USA) was used to examine for HPV DNA in each specimen, including a mixture of probes for cervical cancer associated HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The US Food and Drug Administration approved threshold of 1 pg of HPV DNA/ml of test solution was the positive control. The viral loads were expressed as copies of HPV genomes/100 ng. For the PCR method, MY09-MY-11-HMB01 L1 consensus primers were used in the amplification system, followed by a single hybridization with a reverse line blot detection method (the prototype of Roche linear array HPV test, Roche Molecular Systems Inc, Pleasanton, CA, USA). The HPV genotyping strips contained 29 probe lines plus one reference line, detecting 27 individual HPV genotypes and two concentrations of the β-globin control probe. Two bovine serum albumin (BSA) conjugated probes per HPV probe per HPV type, corresponding to each of two hypervariable regions within the MY09/MY11 amplicon, were deposited in a single line for each of the following HPV types: 16, 18, 26, 31, 33, 35, 39, 42, 45, 51 to 59, 66, 68, MM4, MM7, MM8, and MM9.

Real-time PCR

DNA amplifications were carried out in a 96-well reaction plate format in an ABI Prism 5700 Sequence Detection System. Amplification and quantification of the E2 and E6 genes were performed simultaneously in separate reaction tubes. Both the HPV E2 and E6 PCR reactions were performed in triplicate. Multiple negative water blanks were included in every analysis. The reaction was performed in a 25 µl mixture containing 1x reaction buffer (HPTM HotStart Taq SYBR Green Kit, Cat No. PTM767B, Protech), and 100 nM of primers for both E2 and E6 regions. Fifty nanograms of total DNA were added to the reaction mixture. The primer sequences of E2 and E6 for HPV type 16, 18, 52 and 58 were as shown in Table 3. The amplification conditions were as follows: 10 min at 95°C, a two-step cycle at 95°C for 10s and 60°C for one minute for a total of 45 cycles. The specificity was verified by construction of a dissociation curve using 2% Tris-Borate-EDTA agarose gel electrophoresis. Two standard curves were obtained by amplification of a serial dilution of cloned HPV 16 (from base 28 to base 3890) or HPV 18 (from base 45 to base 3993) DNA fragments or HPV 52 (from base 95 to base 3895) or HPV 58 (from base 45 to base 3994) DNA in pGEM T-Easy vector containing equivalent amounts of E2 and E6 genes from 10 to 10,000,000 copies/µl. The number of

threshold cycles obtained from E2 PCR and from E6 PCR was equivalent in each run. Linear plots of the log of copy numbers vs. the number of the threshold cycle were consistently obtained for both genes, and the correlation coefficient was between 0.995 and 1.00 in each run.

This technique was developed based on the following assumptions: 1) preferential disruption of E2 causes the absence of E2 gene sequences in the PCR product following integration, 2) copy numbers of both genes should be equal to those of the episomal forms, and 3) E2 gene copy numbers will be smaller than that for E6 in concomitant forms.

Concentrations of HPV DNA were expressed as copies of the HPV genome in 50 ng of cellular DNA. Ratios of E2 to E6 of less than one indicated the presence of both integrated and episomal forms. The integrated E6 was calculated by subtracting the copy numbers of E2 (episomal). The ratio of E2 to integrated E6 represented the amount of the episomal form in relation to the integrated form.

Statistical analyses

The 2-year cumulative risks stratified by the change of viral load at the first two time points (viral load increased or not increased) for histologically confirmed HSIL attributable to HPV 16, 18, 52, or 58 were analyzed via the Kaplan-Meier method. Logistic regression analysis was used to calculate the odds ratios (OR) and their 95% confidence intervals (CIs) for HSIL associated with change of HPV load (increased vs. not increased), high viral load ($> 10^5$ vs. $\leq 10^5$), age (≥ 30 vs. <30 years) and repeat Pap smears (abnormal vs. normal). The correlation between HPV loads determined by real-time PCR and HC2 was estimated using Pearson's correlation. The differences in HPV integration status between LSIL and HISL groups were analyzed using Fisher's exact test. All statistical analyses were performed using SAS 9.13.

Chapter 3: Results and Discussions

I. Detection and Quantitation of Human Papillomavirus Type 16, 18 and 52 DNA in the Peripheral Blood of Cervical Cancer Patients

Clinical characteristics of cervical cancer patients

The study sample included 70 patients with cervical cancer. Among them, 10 had stage IA1 disease (microinvasion), 23 had IB, 2 had IIA, 17 had IIB, 5 had IIIB, one had IVB and 12 had recurrence. The mean age of cervical cancer patients was 53 years (range, 33–82 years). Histological diagnoses included squamous cell carcinoma (n = 56), adenocarcinoma (n = 8) and adenosquamous carcinoma (n = 6).

Study I.A. HPV DNA type 16, 18, 52 tests using real-time PCR in cervical swab and blood

A total of 60 patients with invasive cervical cancer were recruited, but samples for only 58 were obtained. Of the 58 patients with invasive cervical cancer whose cervical swabs were available, 33 patients had positive test results for HPV DNA 16, 18 or 52 from cervical swabs. Sixteen out of 33 (48%) patients with cervical cancer were found to have HPV DNA in blood. As shown in Figure 1A, HPV type 16 DNA was found in cervical swabs of 4% (1/25) of the control group, 0% (0/10) of patients with CIN I, 30% (3/10) with CIN II, 25% (5/20) with CIN III, 50% (5/10) with microinvasion, and 36.2% (21/58) with invasive cervical cancer. By contrast, HPV type 18 DNA was found in cervical swabs of 0% (0/25) of the control group, 10% (1/10) of patients with CIN I, 20% (2/10) with CIN II, 0% (0/20) with CIN III, 0% (0/10) with microinvasion, and 15.5% (9/58) with invasive cervical cancer. Type 52 DNA was not (0/25) detected in cervical swabs of patients in the control group, or 30% (3/10) of patients with CIN I, 20% (2/10) with CIN II, 30% (6/20) with CIN III, 20% (2/10) with microinvasion, nor 17.2% (10/58) with invasive cervical cancer.

Of the 60 patients with invasive cervical cancer, 27% (16/60) had positive test results for HPV DNA in blood. By contrast, no patient with microinvasion (0/10), CIN III (0/20), CIN II (0/10), CIN I (0/10) and none (0/25) in the control group had HPV DNA detected in blood. Furthermore, five percent (3/60) of patients with invasive cervical cancer had HPV 16, 16.7% (10/60) had HPV 18 and 8.3% (5/60) had HPV 52 DNA detected in blood

(Figure 1B). Three percent (2/60) of patients with invasive cervical cancer had multiple types (one type 16 and 18, the other type 18 and 52) HPV DNA detected in blood.

Study I.B. Dynamic change of viral loads and median log of HPV DNA type 16, 18 and 52 in cervical swabs

The dynamic change in the median log of HPV DNA viral concentration (copies/genome) detected in cervical swab specimens varied for each subtype of HPV, as shown in Figure 1A. The median values for types 16, 18 and 52 detected in invasive cervical cancer were 1.0×10^5 (range $0.39\text{-}5.73 \times 10^6$), 5.9×10^3 (range $295.22\text{-}1.20 \times 10^7$), and 8.9×10^5 (range $442.14\text{-}3.0 \times 10^9$) copies/μg, respectively. In general, the viral loads of HPV type 16 and 52 increased from CIN to invasive cancer in contrast to that of HPV type 18, which revealed a fluctuating pattern.

Study I.C. Viral loads of HPV 16, 18, 52 in blood of cervical cancer patients and its correlation to clinical outcome

The viral load was higher for both HPV type 52 DNA (1.34-3700 copies/ml in blood with mean value of 771.26) and type 16 DNA (1.035-1756 copies/ml with mean value of 586) than for HPV type 18 DNA (0.003-7.94 copies/ml with mean value of 1.8) (Figure 1B). The viral loads of the different HPV types found in blood and the disease stage and sites are shown in Table 2. The prevalence of HPV DNA detection in blood of cervical cancer patients was not significantly different for each cancer stage ($p > 0.05$; stage IB: 26% vs. IIB: 18% vs. IIIB: 20% vs. IV: 100% vs. recurrence: 45%). All three women with HPV type 16 detected in blood also had HPV type 16 in cervical swabs. Of the ten women who had HPV type 18 detected in blood, six had positive HPV type 18 DNA detected in a cervical swab and one had HPV type 16 detected in a cervical swab, while three had no evidence of HPV in cervical swabs, including one with bone metastasis without local recurrence. Of the five women with HPV 52 detected in blood, four also had type 52 viral DNA detected in a cervical swab, and one had both HPV type 16 and 52 detected in a cervical swab. Seven of the 12 HPV-positive blood samples from primary invasive cervical cancer patients had bulky tumors (> 4 cm). Six of the 12 HPV-positive blood samples from invasive cervical cancer patients showed abnormal SCC levels (> 2.5 ng/mL).

Among the 16 patients with HPV DNA detectable in blood before treatment, seven

had stage IB disease, three had stage IIB, one had stage IIIB, one had stage IVB, three had stage IB disease with distant recurrence (two bone metastases and the other abdominal metastases), and one had stage IIB with pelvic recurrence.

Study I.D. Follow-up of HPV DNA in blood of cervical cancer patients

HPV DNA in blood was detected during follow-up in those three patients with stage IB disease with distant recurrence. One patient with stage IIB with pelvic recurrence had undetectable HPV DNA in blood after treatment and was free of disease. Among the remaining 12 patients with invasive cervical cancer, three patients (two initially had stage IB and one initially had stage IIIB disease) developed recurrence at 5 months, 6 months and 8 months after treatment. In addition, two had persistent disease (one initially had stage IIB with para-aortic lymph node metastasis confirmed by fine needle biopsy under CT guidance, and the other initially had stage IVB disease). HPV was detected in blood after treatment of all 5 patients. At 3 months after treatment, eight out of ten patients with cervical cancer who had HPV DNA detected in blood had recurrence (including two with persistent disease). Among these eight patients, seven (87.5%) had distant metastases. The site of distant metastasis was bone in two, abdomen in three, neck lymph nodes in one and vulva in one. Of the remaining two patients, one was negative for HPV in blood 9 months after treatment and was clinically free of disease, while HPV DNA was still detected in blood in the other patient who was clinically free of disease at the last follow-up visit. The other six cervical patients with negative HPV DNA results in blood after treatment showed no recurrence up to December 31, 2004, a median follow-up duration of more than 18 months.

I.E. Discussion

The results of this study confirm that HPV DNA can be detected and quantified using real-time PCR from blood samples of patients with invasive cervical carcinoma. Circulating HPV DNA was not present in controls with benign tumors, or patients with CIN lesions or microinvasive cervical cancers. Moreover, type 52 HPV DNA was prevalent in the blood of 8.3% and in the cervical swab of 17.2% of invasive cervical cancer patients. Unfractionated whole blood was used to test for HPV because it combines all blood components that can harbor HPV. This might provide a more accurate estimate of the absolute viral loads in the blood circulation of patients. At the beginning of the study, HPV viral loads were compared between plasma and whole blood, but found no

significant difference between these two measurements (data not shown). Use of whole blood has the additional advantage of being collected simply and in a uniform specimen size.

Only one study using real-time PCR for detection and quantitation of HPV DNA in the plasma of patients with cervical cancer has been previously reported. In that study, Dong et al found HPV 16 or HPV 18 E7 DNA in only 6.9% of invasive cervical cancers by conventional PCR (Dong et al, 2002). The authors stated that major limitations in their study included the retrospective nature of the analysis subject to selection bias and lack of detailed follow-up information. Here, data is provided from a prospective study with HPV DNA detection at the time of diagnosis and at follow up after treatment. Because low viral loads of HPV DNA in blood may be detectable by real-time PCR, use of this method may improve detection rates compared to conventional PCR. More than one-fourth (27%) of patients with invasive cervical cancer had HPV DNA detected in their blood samples. Approximately 50% of patients with confirmed HPV 16, 18 or 52 positive cervical cancers had HPV DNA detected in their blood using this study's assay system. These results suggest that this assay is superior to those of other reports (Dong et al, 2002; Pornthanakasem et al, 2001; Liu et al, 2001). Moreover, this study also provides serial follow-up data on HPV DNA viral load in cervical cancer patients after treatment, suggesting its prognostic significance.

Although the exact pathway by which tumor DNA is released into the bloodstream remains unclear, its presence and quantity are likely to be a reflection of tumor load or metastasis and may have prognostic value for patients with cancer (Castells et al, 1999; Sidransky, 2000; Jen et al, 2000). Because the life cycle of HPV occurs entirely within epithelial tissues, the virus is not usually found in the bloodstream. A possible hypothesis to explain the presence of circulating viral DNA detected in patients may be due to the lysis of circulating cancer cells (necrosis or apoptosis) or micrometastases (active release of tumor DNA) shed by the tumor (Jahr et al, 2001). Circulating cervical cancer cells have been found by analyzing HPV specific mRNA in peripheral blood cells of advanced stage cervical cancer patients with metastasis (Tseng et al, 1999). Detection of HPV DNA in blood may be an early sign of distant cervical cancer metastasis. Thus, even in the absence of active cell shedding, DNA and proteins could be translocated from neoplastic cells to a distant site via the bloodstream.

In this study, six cervical cancer patients negative for HPV DNA after treatment showed no recurrence during follow-up. During follow-up, eight out of ten cervical cancer patients with HPV DNA detected in the blood at 3 months after treatment had either recurrence or failure of treatment. Among them, seven of eight patients had distant metastases. There was no significant difference in HPV DNA detection rates in the early or advanced stage at the time of diagnosis. Besides, three of four patients with distant metastasis did not have increased tumor markers at the time of diagnosis. These findings may suggest that distant metastasis is related to persistent detection of HPV DNA after treatment, supporting the idea that circulating HPV DNA may play a crucial role in metastasis. Therefore, detection of HPV DNA in blood could be a specific, although not very sensitive, genetic marker that has a strong association with cervical cancer metastasis. Detection of circulating HPV DNA after treatment could also predict recurrence. It is postulated that detection of HPV DNA in blood might be a useful marker to select subsets of patients who need more aggressive treatment.

Cervical cancer is the most common female gynecologic cancer in Taiwan and has a high mortality rate. With HPV vaccination studies currently underway, it is important to map the epidemiology of HPV infection in populations worldwide to facilitate the implementation of future prevention programs. Previous studies have reported that HPV types 52 and 58 seem to play a more prominent role in cervical cancer in Asia than HPV types 31, 33 and 45, which are more common in western countries (Liaw et al, 1995; Huang et al, 1997; Clifford et al, 2003; Pirami et al, 1997). The distribution of various HPV types reveals a more prominent role of HPV 52 in Taiwan than in other parts of the world. Among the Taiwanese patients of this study, HPV 16 (36.2%), 18 (15.5%) and 52 (17.2%) were the most common HPV types in invasive cervical cancers, together accounting for almost 70% of the samples. Moreover, HPV type 52 DNA was detected in the blood of 8.3%, HPV type 16 in 5%, and HPV type 18 in 16.7% of invasive cervical cancer patients. The high prevalence of HPV 52 in the blood was unexpected. The assay was also validated with HPV 52 E6 primer containing 106 bp (data not shown), confirming that there was no misclassification. In general, the viral load of HPV type 16 and 52 increased as cancer stage increased from CIN to invasive cancer. This was in contrast to findings for HPV type 18 which revealed fluctuating viral loads with cancer stage. The clinical data revealed that two patients with invasive cervical cancer and heavy

viral loads of HPV type 52 (> 10^7 copies/genome) died within 8 months. Three patients with HPV type 52 DNA detected in blood before treatment were found to have undetectable blood levels after treatment and were clinically free of disease at the last follow-up. Of the two patients with HPV type 52 DNA detected in blood after treatment, one initially had stage IIIB disease with neck lymph nodes recurrence and the other had persistent stage IVB disease.

The detection rate for HPV type 18 in this study was higher than that of other reports. In the patients, most of the detectable viral loads for HPV type 18 were very low (< 1 copy/ml). All positive real-time PCR products were confirmed, including those for HPV type 18 by direct sequencing, and tested with CaSki and HeLa cell lines bearing a known amount of HPV genomes. It was found that six of ten patients with HPV DNA 18 detectable in blood developed recurrence.

In this study, an assay was developed to detect and quantitate viral loads of HPV DNA using real-time PCR, which is a rapid, sensitive and specific method. The same HPV type was detected in the blood and genital tract of 12 of 16 (75%) women whose blood specimens were either HPV 16, 18, or 52 positive. The reason for the discrepancy between findings for cervical swabs and blood samples observed in the remaining 4 women whose blood specimens were HPV type 18 could not be determined. However, data was confirmed using different primers and methods. One possible reason for this discrepancy may be that virus from the genital tract was eliminated after treatment, or it may be that only one type of viral load was detected in the genital tract in patients with multiple latent infections of different HPV types. Dong et al also reported the same HPV type was detected in the plasma and genital tract of 9 of 14 (64.3%) women whose plasma specimens were HPV 16/18 positive (Dong et al, 2002). Contamination was considered unlikely because all HLA-DQα gene negative blood samples were also HPV DNA negative. All PCR negative controls were also negative.

There are a number of limitations to the present analysis. First, the patients were studied for a limited period of time and the sample size was small. Extended follow-up and study of greater numbers of patients may be required before a definite conclusion regarding the usefulness of blood HPV DNA to detect tumor recurrence and its impact on the survival of patients can be reached.

II. Integrated Human Papillomavirus Type 52 and 58 Infrequently Found in Cervical Cancer, and High Viral Loads Predict Risk of Cervical Cancer

Clinical characteristics of enrolled patients

Cervical swabs were obtained from 178 patients including 81 with cervical cancers and 97 with CIN II-III lesions. Among the 81 cervical cancer patients, disease was stage IA in 9, IB in 35, IIA in 3, IIB in 24, IIIA in 1, IIIB in 6, and IV in 3. The median age of patients with cervical cancer was 56.5 years (range, 30-85 years) and of patients with CIN II-III it was 44 years (range, 25-84 years) ($p<0.001$). Histological diagnoses included squamous cell carcinoma (n=67), adenocarcinoma (n=11) and adenosquamous (n=3).

Study II.A. HPV DNA typing using genechip method

Genechip analysis detected HPV DNA sequences in 87.7% (71/81) of the cervical cancer swabs, among which 76.1% (54/71) harbored a single type and 23.4% (17/71) contained multiple types. HPV 16 was detected in 30.9% (25/71), followed by type 52 in 16.1% (13/71), type 18 in 14.8% (12/71) and type 58 in 12.4% (10/71). In the swab samples from patients with CIN II-III, HPV DNA was detected in 83.5% (81/97), among which 64.2% (52/81) harbored a single viral type while 35.8% (29/81) harbored multiple types. The prevalence of HPV type 16, 18, 52 and 58 in CIN II-III were as follows: 19.6% (19/81) for HPV type 58, followed by 18.6% (18/81) for HPV type 16, 18.6% (18/81) for HPV type 52 and 7.2% (7/81) for HPV type 18. All swabs that were positive for HPV 16, 18, 52 and 58 detected in genechips were quantified by real-time PCR. The discrepancy between HPV detection in cervical cancer using genechips versus real-time PCR analysis (E6 level undetectable) for each of the different DNA types was as follows: 8% (2/25) for type 16, 0% (0/12) for type 18, 7.7% (1/13) for type 52 and 20% (2/10) for type 58.

Study II.B. Detection of physical status in HPV 16, 18, 52 and 58 DNA

Differences in the prevalence of the physical status of HPV 16, 18, 52 and 58 DNA detected in the swabs of cervical cancer patients are shown in Figure 2. The prevalence of integrated HPV type 16, 18, 52 and 58 DNA detected in swabs from patients with CIN II-III was as follows: 92.3% for HPV type 52, 45.5% for HPV type 16, 42.9% for HPV type 18, and 12.5% for HPV 58. A significant difference in the physical status of HPV 16,

18, and 52 DNA was found between patients with CIN II-III and cervical cancer (p=0.022 for type 16, p=0.022 for type 18 and p<0.001 for type 52). However, integrated viral DNA of type 16 and 18 was more prevalent in cervical cancer compared to CIN II-III, while cervical cancer swabs showed less prevalent HPV type 52 and similar prevalence of HPV type 58 (Figure 3).

Study II.C. The median and median log of E2, E6 viral loads of HPV 16, 18, 52 and 58 DNA in CIN II-III and cervical cancer

Differences in the median log of E6 viral loads of HPV DNA type 16, 18, 52 and pooled data between CIN II-III and cervical cancer are shown in Figure 3. The median E2 and E6 viral loads for pooled data of HPV 16, 18, 52 and 58 DNA showed either significant or marginally significant differences between patients with early cervical cancer and patients with advanced cervical cancer (p=0.029 and p=0.054, respectively).

When the median log of E6 viral loads was used to predict which patients had cervical cancer, the cut-off value of 4.15 for the median log of the viral load of HPV 16 achieved 62.5% sensitivity with a 25% false positive rate. By contrast, the cut-off value of 3.76 of the median log of the viral load of HPV 18 achieved 83.3% sensitivity with a 0% false positive rate, and that of 4.92 of the median log of the viral load of HPV 52 achieved 75% sensitivity and a 6.3% false positive rate.

Study II.D. ROC curve predicts the assignment to CIN II-III or cervical cancer groups

The ROC curve analyses indicated that the area under ROC curve was 73.8%, 92.9%, and 88.5%, suggesting that the model could accurately predict the assignment to CIN II-III or cervical cancer groups in 73.8%, 92.9%, and 88.5%, of patients with positive HPV type 16, 18 and 52 DNA, respectively. The ROC curve of the median value of the log of E6 viral loads for each type or pooled data of HPV 16, 18 and 52 DNA could predict progression of disease from CIN II-III to cervical cancer ($p\leq$0.002 for all tests of AUC>0.5). These ROC curves are shown in Figures 5, 6, 7 and 8.

II.E Discussion

Worldwide, cervical cancer is the second most common malignant disease among women. The causal link between human papillomavirus (HPV) and cervical cancer is now

established beyond doubt, and the association has become an important model of viral carcinogenesis. Cervical cancer is the leading cancer among women in Taiwan. A Taiwan Cooperative Oncologic Group (TCOG) study reported by Chen et al, 2006 confirmed the high prevalence and risky nature of HPV 52 and 58 in the Taiwanese population. However, in Taiwan, only limited data describes the pattern of HPV genotype distribution in preinvasive lesions or invasive cervical carcinoma. More data are needed for the understanding of the epidemiology of HPV infection in the population and the implications to vaccine development. Currently two sets of prophylactic vaccines undergoing clinical trials do not include HPV types 52 and 58. Both HPV types should be considered in the strategies of new HPV vaccine development.

There are HPV and non-HPV factors that contribute to HPV-induced malignant progression. Non-HPV factors are necessary for the development of cervical cancer such as multiple sexual partners, smoking, hormones, immunosupression, genetic predisposition, etc. Viral factors include HPV high-risk type, viral variants (non-European type), high viral loads and HPV integration. High risk HPV DNA is a marker for current or subsequent development of precursor lesions. Persistent HPV DNA type-specificity is an even stronger predictive factor. Testing for HPV oncogenic activity, rather than for the presence of HPV DNA, may therefore be a more relevant clinical indicator of cervical lesions and cervical cancer. The detection of HPV E6 DNA viral loads and HPV integration may be used as a clinically predictive marker to identify women at risk of developing high-grade cervical dysplstic lesions and cervical carcinoma. It has been reported that high HPV viral loads in cervical samples are associated with an increased risk of squamous intraepithelial lesions and invasive cervical cancer (Josefsson et al, 2000; Sun et al, 2005).

The integration of HPV DNA into the host genome is thought to occur early in cancer development and to be an important event in malignant transformation of cervical cancer (Boshart et al, 1984; Vernon et al, 1997; Nagao et al, 2002). However, most studies on the integration of HPV DNA focused on type 16 and a few on type 18 or other oncogenic types such as 31 and 35 (Choo et al, 1987; Jeon et al, 1995; Boshart et al, 1984; Vernon et al, 1997; Peitsaro et al, 2002; Nagao et al, 2002; Badaracco et al, 2002; Das et al, 1992; Pirami et al, 1997). While HPV type 52 and 58 are oncogenic types with relatively low prevalence in cervical cancer in the Americas, Europe, Africa and Southeast Asia, they are

as prevalent in Taiwan and other Asian countries as the known high-risk (for cervical cancer) HPV types 16 and 18 (Walboomers et al, 1999; Liaw et al, 1995; Huang et al, 1997; Tseng et al, 1999).

To the best of our knowledge, the prevalence of integrated HPV 52 and 58 DNA in patients with CIN II-III and cervical cancer has not been reported. In this study, the prevalence of integration of HPV type 52 and 58 in patients with cervical cancer was unexpectedly low, while the prevalence of integration of HPV type 52 in patients with CIN II-III was very high (92.3%). These results indicate that integration of HPV type 52 is not required for progression from CIN II-III to cervical cancer. On the contrary, detection of a high E6 viral load of HPV type 52 detected in cervical cancer patients was a good marker of invasiveness. This finding reflects that unknown factors other than integration are likely to be involved in the induction or maintenance of E6 viral loads leading to the expression of E6 oncoprotein in the carcinogenesis of cervical cancer. The high prevalence of intact HPV episomal E2 DNA supports that disruption of ORF is not required for increased oncogene expression in patients with HPV type 52 and 58. This study highlights the need for investigating alternative mechanisms of oncogene expression during the oncogenesis of HPV 52 and 58.

There are several possible explanations for the presence of episomal HPV DNA 52 and 58 in most of the swab samples of cervical cancer in this study. These include extensive sequence variations present in the HPV 52 and 58 genes, mutations in the long control region which may serve as an alternative mechanism for increasing the expression of viral oncogenes, and epigenetic events such as methylation of viral DNA. A significant proportion (10-30%) of invasive cervical cancers containing only episomal HPV DNA have been reported, demonstrating that HPV 16 integration might not be essential for malignant transformation (Badaracco et al, 2002; Das et al, 1992). In this study, 17.5% of invasive cervical cancers contained only HPV type 16 episomal HPV DNA, a finding which is compatible with previous studies (Badaracco et al, 2002; Das et al, 1992). Elimination of the expression of HPV 16 E2 protein may be due to integration or to up-regulation of the transcription of the E6 and E7 oncogenes resulting from YY1 mutation, possibly providing a selective growth advantage for the infected cell (Park et al, 1999; Dong et al, 1994). This may contribute to an increase in cell proliferation and genomic instability, leading to further genetic alterations (Alazawi et al, 2004). The E6

and E7 oncoproteins interfere with the normal cell cycle by targeting the p53 and pRb tumor suppressor proteins, respectively. These effects are important steps in cervical carcinogenesis, resulting in the loss of cell cycle control (Dyson et al, 1989; Bremer et al, 1995).

Identification of viral and host factors that contribute to the delineation of women at the highest risk of HPV progression to cervical neoplasia is an essential step in determining the candidate markers of potential use for diagnostic or prognostic purposes. The potential utility of viral load measurements as etiologic risk factors as well as diagnostic and prognostic tools for cervical cancer in clinical practice warrants their continued attention in research. Previous cross-sectional epidemiologic studies (Josefsson et al, 2000; Dalstein et al, 2003) and one longitudinal study (Dalstein et al, 2003) have demonstrated an association between increased HPV viral load and risk of carcinoma *in situ* (CIS) and cervical cancer. However, its predictive utility remains unclear and a model establishing a useful cut-off value for identifying the subgroup of patients with cervical cancer has yet to be determined. Viral load has been suggested as a marker for non-transient infection, and high HPV load in smears with normal cytology has been associated with increased risk of developing dysplasia and CIS (Josefsson et al, 2000; Moberg et al, 2004). However, it remains unclear whether high viral load is also predictive of invasive cervical cancer, as past studies have pooled the outcomes of invasive cancers together with earlier stages of the disease and data is limited for HPV types other than 16 and 18 (Moberg et al, 2005; Lorincz et al, 2002; Gravitt et al, 2003).

Because most studies have focused on HPV 16 or overall (i.e., non-type specific) viral load measures, extremely little is known about the relationship of viral load to viral types other than HPV type 16, such as HPV type 52 and 58, and their relationship to cervical neoplasia. Moberg et al reported that high loads of HPV type 16 and 18/45 are type-dependent risk markers for invasive cancer (Moberg et al, 2005). By contrast, whether high loads of HPV 52 and 58 increases the risk of cervical cancer has not been previously reported. As a consequence, the association between viral load and development of CIS may not automatically imply a strong association of high viral load and progression into invasive carcinoma. The results indicate that elevated viral loads of HPV 16, 18 and 52 in cervical swabs is associated with increased risk of invasive cervical cancer. The data also indicated that the median E2 and E6 viral loads for pooled data of all

subtypes of HPV 16, 18, 52 and 58 showed either significant or marginally significant differences between patients with early cervical cancer and patients with advanced cervical cancer ($p=0.029$ and $p=0.054$, respectively). Potential clinical applications of viral load measurements include use as an indicator of a missed cervical cancer lesion among CIN II-III lesions with margin involvement or an undetected advanced cervical cancer needing more aggressive therapy. High viral loads of HPV in cervical swabs may be indicative of a HLA class II-mediated immune response to HPV, reduced immune surveillance against HPV infection at mucosa, and subsequent development of cervical cancer (Beskow et al, 2005). Longitudinal studies including both physical state and load of the virus are needed to determine their relative importance in the development of cervical cancer.

Measurement of HPV DNA load has been suggested as having positive predictive value for detecting CIN III and cancer based on the assumption that higher load values are more strongly associated with severe disease (Josefsson et al, 2000; Lorincz et al, 2002; Sherman et al, 2002). HPV load determined using the Hybrid Capture 2 (HC2) DNA test has been shown to be linear over a broad dynamic range and well correlated using quantitative PCR measurements (Lorincz et al, 2002; Sherman et al, 2002). However, Hybrid Capture 2 does not adjust load determinations for specimen cellularity and cannot distinguish between infections with single or multiple types of HPV. The value of viral load estimates based on summary measurements of HPV, such as those generated by the HC2 assay, may be limited in not distinguishing HPV types, or multiple infections. Within any specific grade of CIN, infections with multiple oncogenic types were associated with significantly higher HPV loads using Hybrid Capture 2 than infections with single types. However, after adjusting for number of HPV types detected, HPV load would not have specifically identified patients with CIN III (Lorincz et al, 2002). Several studies have noted differences in viral load pattern between HPV 16 and other HPV types (Moberg et al, 2004; Lorincz et al, 2002). Gravitt et al found an increased odds ratio (OR) of HSIL/cancer with HPV 16 load, but did not find a similar trend for HPV 18 (Gravitt et al, 2003). These observations led to the investigation of the risk of cervical disease associated the individual high risk HPV types.

Approximately 35% of CIN II-III lesions in this study were infected with multiple oncogenic HPV types. The relationship was examined between viral load and CIN II-III

or cervical cancer for high-risk HPV types 16, 18, 52 and 58, which accounted for about 70% of HPV types. The E6 viral loads of both single and multiple infections with HPV 16, 18 and 52 DNA showed a strong positive relationship with cervical cancer. However, HPV 58 load appeared to have a weak relationship with risk of cervical cancer.

Real-time PCR is a recent addition to the molecular diagnostic methods used to detect HPV infection. Real-time PCR has the advantage of being highly specific, reproducible, and capable of detecting HPV viral load at up to eight orders of magnitude in a linear range. These qualities make real-time PCR attractive for use in epidemiological studies and as a potential diagnostic test. Several studies reported the predictive value of HPV viral burden was demonstrated using real-time PCR. These studies, however, only focused on the quantitation of HPV type 16 viral loads (Lorincz et al, 2002; Lefevre et al, 2004). Due to wide variation in viral load among patients, a model for measuring viral load with cut-off values to predict severity of disease remains to be established. In this study, the median log of the viral load of HPV 16, 18 and 52 DNA was successfully used to predict the presence of cervical cancer. The selected cut-off values of the median log of the viral load in HPV 16, 18 and 52 DNA achieved 62.5-75 % sensitivity and 0-25 % false positive rates in predicting the presence of cervical cancer. The ROC curve analyses indicated that the model could accurately predict the diagnostic group of CIN II-III or cervical cancer in 73.8%, 92.9%, and 88.5% of patients with positive HPV 16, 18 and 52, respectively.

There are a number of limitations to the present study. First, the sample size is small, and the study design was cross-sectional. A longitudinal study involving a greater number of patients is required before definite conclusions can be reached regarding the usefulness of HPV DNA viral loads to diagnose and predict the risk of cervical cancer. This study shows that quantification of E6 HPV 16, 18 and 52 DNA can serve as a prognostic tool for identifying women who are at increased risk of developing cervical cancer. Further longitudinal studies are needed to establish the predictive significance of viral load measurements for the different oncogenic HPV types.

III. Human Papillomaviral Load Changes in Low-Grade Squamous Intraepithelial Lesions of the Uterine Cervix

Clinical characteristics of enrolled patients

Among the 294 women with LSIL having at least four follow-up visits in 3 months, 76.9% were HPV DNA positive by PCR. The frequencies of the major, baseline HPV genotypes were: HPV 16 (4.8%), 18 (2.4%), 52 (10.9%), 58 (4.1%), 53 (8.5%), and 51 (6.8%). The prevalence of HPV 16, 18, 52, and 58 in HSIL were as follows: 31.7% (92/290) - type 16, followed by 20.0% (58/290) - type 58, 19.7% (57/290) - type 52, and 1.7% (5/290) - type 18.

Study III.A. Median E6 viral loads

For the 61 eligible women with LSILs, the clinical characteristics and median viral loads are shown in Table 1. The median viral loads of HPV types 16 and 52 among women with LSILs and HSILs differed significantly ($p = 0.049$ for HPV 16 E6, $p = 0.032$ for HPV 52 E6). The median viral loads of HPV types 18 and 58 among women with LSILs and HSILs did not differ significantly ($p = 0.499$ for type 18, $p = 0.184$ for type 58). The median viral load ranged from 1.3×10^4 to 3.7×10^4 copies per 100 ng of DNA. Therefore, high viral loads were defined as those greater than 10^5 copies per 100 ng of DNA. The HPV 16, 18, 52, and 58 viral loads tested by real-time PCR varied from 2.4×10^2–7.6×10^6 copies per 100 ng of DNA, which is equivalent to 15,151 cells. HPV 16, 18, 52, and 58 loads tested by HC2 varied from 5.1×10^3–1.1×10^8 copies per 100 ng of DNA, which is also equivalent to about 15,000 cells. Women who had high viral loads ($> 10^5$ copies) versus low viral loads ($\leq 10^5$ copies) at baseline had marginally significant increased risk of disease progression ($p = 0.07$).

Study III.B. Two-year cumulative risk with dynamic change of HPV

Overall, 61 women after cytological examination with LSILs attributable to HPV 16, 18, 52, or 58 had a 2-year cumulative risk of developing HSILs of 21.5%; 29.3% (4/14) with HPV 16 (OR = 1.5, 95% CI = 0.4 to 5.8, $p = 0.34$), 12.5% (1/8) with HPV 18 (OR = 0.6, 95% CI = 0.1 to 5.3, $p = 0.23$), 26.9% (7/26) with HPV 52 (OR = 0.7, 95% CI = 0.2 to 2.6, $p = 0.34$), and 7.7% (1/13) with HPV 58 (OR = 0.3, 95% CI = 0.0 to 2.1, $p = 1.61$) (Figure 9).

Based on viral load changes detected using real-time PCR, women with LSILs who had viral load increases for HPV 16, 18, 52, or 58 between baseline and a 6 month follow-up had a 2-year risk of developing HSIL of 45%; seven-fold greater than those without increases in viral loads (OR = 7.6, 95% CI = 1.9 to 24.3, $p < 0.01$) (Figure 10). Overall, women with LSILs and persistent HPV infections had a 2-year cumulative risk of developing HSILs of 25%, compared to 11.5% for those with transient infections (OR = 2.5, 95% CI = 0.5 to 12.7, p = 0.27) (Figure 11).

Women with LSILs due to HPV 16, 18, 52, or 58, who had increases in viral loads between baseline and 6 month follow-up had risk of persistent abnormal Pap smears 45-fold greater than those without increased viral loads (OR = 45.6, 95% CI = 6.4 to 326.0, $p < 0.001$).

Using logistic regression for modeling changes in viral load data, repeat abnormal Pap smears, and patient age at enrollment, women with LSILs who had viral load increases between baseline and 6 month follow-up for HPV 16, 18, 52, or 58 had increased risk for HSILs, as tested by real-time PCR (OR = 8.3, 95% CI = 2.0 to 34, $p < 0.01$) or HC2 (OR = 5.6, 95% CI = 1.5-21.4, $p < 0.01$). In addition, women with LSILs, repeat abnormal Pap smears (OR = 2.1, 95% CI = 0.5 to 8.3, $p > 0.05$), and aged younger than 30 years (OR = 1.8, 95% CI = 0.3 to 11.7, $p > 0.05$) did not show an increase in risk of developing HSILs (Table 5).

Study III.C. Comparison of HPV viral load by HC2 and real-time PCR

Real-time PCR and HC2 correlated well for determining viral load change (increased or not increased) at baseline and 6 month follow-up to predict disease progression. Based on viral load change tested by HC2, women with LSILs due to HPV 16, 18, 52, or 58 who had viral load increase between baseline and 6 month follow-up had a risk of developing HSILs of 44%; six-fold greater than those without increased viral loads (OR = 6.1, 95% CI = 1.6–22.7). This result is consistent with real-time PCR of 45%; seven-fold greater than those without increased viral loads by real-time PCR. The logarithm of HPV 16, 18, 52, and 58 viral loads estimated by real-time PCR were plotted as functions of the logarithm of HPV viral loads estimated by HC2. The two measurements for viral load correlated well (Pearson's correlation coefficient *rho* = 0.69, $p < 0.001$).

Study III.D. Physical status of HPV 16, 18, 52, and 58 DNA in women with LSILs and longitudinal follow-up

The prevalence of both integrated and episomal (mixed type) infections for women with LSILs at baseline for HPV 16, 18, 52, and 58 were as follows: 7.1% (1/14) - type 16, 12.5% (1/8) - type 18, 7.7% (2/26) - type 52, and 0% (0/13) - type 58. No pure single type of viral integration was found in women with LSILs due to HPV 16, 18, 52, and 58 at baseline or during follow-up. Integrated and episomal HPV 16, 18, 52, or 58 DNA was detected in only 1 of 13 (7.7%) specimens in progression among women during transition from LSILs to HSILs. The prevalence of integration of HPV 16, 18, 52, or 58 DNA between women with LSILs and HSILs increased significantly ($p < 0.001$). A significant difference or trend in the physical status of HPV 52, 18, and 58 DNA was found between patients with LSILs and HSILs ($p < 0.001$ for type 52, $p = 0.067$ for type 18, and $p = 0.065$ for HPV 58). However, no significant difference in the physical status of HPV 16 DNA was found between patients with LSILs and HSILs ($p = 0.46$). When the comparison was confined to tissue proved HSIL cases, the prevalence of integration of HPV 16, 18, 52, or 58 DNA between LSIL and HSIL cases was still significantly increased ($p < 0.001$). A significant difference or trend in the physical status of HPV 52 or 58 DNA was found between patients with LSILs and HSILs ($p < 0.001$ for type 52, and $p = 0.082$ for HPV 58). However, the physical status of HPV 16 and 18 DNA was not significantly different between patients with LSILs and HSILs ($p = 0.3$ for type 16 and $p = 0.22$ for type 18).

III.E. Discussion

In some developed countries cytologic screening programs using Papanicolaou (Pap) smears have reduced cervical cancer incidence and mortality. Yet, cervical cancer is still one of the most common malignant diseases and leading cause of morbidity and mortality among women worldwide (Parkin et al, 2001). Single Pap tests suffer from suboptimal sensitivity, limited reproducibility, and many equivocal results (Solomon et al, 2001). To compensate for these deficiencies a screening program with repeated testing combined with follow-up of positive cases is necessary. Furthermore, difficulties predicting which cervical lesion will progress, and the high rate of regression of low-grade lesions detected in cytologic screening, emphasizes the need for additional prognostic and diagnostic markers for detection of cervical cancer precursors.

HPV is a common virus infection among women, particularly in younger age groups, and most infections are transient and asymptomatic. Patients with persistent infections with these HPV types have a clearly enhanced risk of developing cervical carcinoma. Large scale of screening studies have shown HPV testing is more sensitive than cytology methods for the detection of high grade cervical lesions (Schiffman et al, 2000). However, the low specificity of current assays and commercial kits hampers the use of HPV testing in screening. A combination of HPV DNA detection and cytology is more suitable for risk assessment of progression to CIN grade III and carcinoma than cytology alone (Sherman et al, 2003). Hence the detection of integrated HPV DNA of HR HPV types for the development of HSIL might serve better as a risk evaluation factor than simple DNA detection. The same would be true for the progression to cervical carcinoma.

In the study population of women with LSILs, viral loads for HPV 16, 18, 52, and 58 increased during the 6 month follow-up after baseline values were obtained. This increase in viral load was associated with a high risk of developing HSILs over a cumulative 2-year period, a seven-fold higher risk estimated by real-time PCR and a six-fold greater risk by HC2 compared to patients whose viral loads did not increase during the follow-up period.

A 45%, 2-year cumulative risk was observed of clinically relevant HSIL for HPV 16, 18, 52, and 58 in women with LSILs and whose viral loads increased between baseline and 6 month follow-up. Moreover, women with LSILs who had viral load increases between baseline and 6 month follow-up for HPV 16, 18, 52, or 58, had risk of persistent abnormal Pap smears; 45-fold greater than did those who had no increase in viral loads (OR = 45.6, 95% CI = 6.4 to 326.0, $p < 0.001$). The frequent monitoring of these women allowed an early diagnosis of high-risk HPV, and thereby, early therapeutic intervention. Indeed HR HPV and especially HPV 16, 18, 52, and 58 infections persisted and progressed throughout the follow-up period. Van Duin et al (2002) reported similar results and hypothesized that increasing viral loads over time would favor viral persistence, and the development of cervical CIN II-III or worse.

Women with LSIL cytology who were HPV 16 DNA positive at baseline did not have significantly increased risk of developing HSIL (OR = 1.5, 95% CI = 0.4 to 5.8).

One possible explanation for this is the number of women with LSILs due to HPV 16 was limited (only 9%), compared to 21% in Castle et al (2005). It was observed that high viral loads (> 10^5 copies) at baseline reached marginal significance (p = 0.07) in predicting disease progression among women with LSILs, consistent with Castle et al (2005). Additionally, neither repeat abnormal Pap smears nor age older than 30 years increased the risk of HSIL. Thus, increased HPV 16, 18, 52, and 58 viral loads was the single most important risk factor for HSIL in the study population.

The increase of viral load detected by both HC2 and real-time PCR methods predicted disease progression to HSIL, with HPV 16, 18, 52, and 58 viral load detection correlating well (only 6% of tested specimens had mixed HPV types) and similar to Prétet et al (2004). The real-time PCR method for E6 HPV 16, 18, 52, and 58 quantifications were used to determine whether the HC2 assay could be suitable as a quantitative viral load assay for clinical practice. Recent data suggests that HR HPV load would help to identify women at high risk for progression to HSIL (Josefsson et al, 2000; Lorincz et al, 2002). However, due to multiple infections, cross-reactivity of non-oncogenic HPV types, and variability of cellularity of cervical scrapings, HC2 assay of HR HPV load has not been validated as a quantitative test.

Although, specimens with HPV 16, 18, 52, and 58 represent about 43% of all LSILs (Chen et al, 2006), they represented 73% of HSILs in the cohort study. Because of the relatively small proportion of HPV 16 in women with LSILs in Taiwan, it seems that distinguishing the risk of cervical precancer among HPV 16-positive women from the lower risk posed by other oncogenic HPV types might have limited clinical value. Alternatively, repeat HPV DNA testing 6 months after baseline testing to determine changes in viral load (increased or not increased) could predict progression of disease among women with LSILs due to HPV types 16, 18, 52, and 58, and might be clinically practical and valuable. Although, it is useful for ASCUS triage (Solomon et al, 2001), HC2 is not recommended for LSIL triage because the high proportion of positive results makes it uninformative (ALTS, 2000; Solomon et al, 2001). Nonetheless, if the elevated risk of HSILs in patients who are positive for HPV 16, 18, 52, or 58 and have viral load increases over the first two time points at 6 month intervals (33% of patients who are positive for HPV 16, 18, 52, or 58) warrants more aggressive treatment, then determination of viral load change (increase) of individual HPV 16, 18, 52, and 58 types

might be useful for the management of women with LSILs. It is noteworthy that women who had undetectable HPV or viral loads that decreased between baseline and a 6 month follow-up still had a 6% (2/34) risk of developing HSILs (data not shown). These data highlight that no single test or combination of tests will provide perfect negative reassurance of cervical precancer or cancer.

Several authors also note that the quantitative measurement of HPV DNA, as an evolutional predictor or determinant of disease severity, is of greater value than a single HPV positive result. For example, the amount of HR HPV DNA increases the grade of the lesion. This increase was noted for HPV 16, but not for HPV 18, 31, 33, 45, or 56 (Pirami et al, 1997). Moreover, the highest HPV 16 loads are predictive of the appearance of carcinoma *in situ* before any intraepithelial lesions are detectable (Josefsson et al, 2000). In a model of the temporal relationship in HPV-induced carcinoma, Peitsaro et al (2002) proposed that the probability of HPV DNA integration increases with high HPV 16 DNA load. In this study, the median viral loads of HPV type 16 and 52 in women with cytologies between LSIL and HSIL differed significantly to those of HPV types 18 and 58. The viral loads between women with integration in LSILs and women with episomal in LSILs could not be compared because of the low integration rate in LSILs cases. However, the median viral loads of E6 between women with integration in primary HSILs and women with episomal in primary HSILs were compared. There were no significant differences in pooling data (including HPV 16, 18, 52 or 58) or type difference between these two groups of patients. (Data not shown.)

The value of 1 pg of HPV genome/ml, which represents the HC2 assay threshold, corresponds to 10^5 copies of HPV genome/ml. Since the cervical samples harbored a mean of 5×10^5 cells/ml, the HC2 cut-off represents 10^5 HPV/5×10^5 cells. Van Duin et al (2002) proposed that it was 2.4×10^4 HPV 16/scrape (one scrape contains approximated 0.6×10^5–2.2×10^5 cells) for women with normal cytologies and 4.3×10^6 HPV 16/scrape for women with abnormal cytologies (Van Duin et al, 2002). The real-time PCR and HC2 measurements of viral load correlated well, even without cell normalization in this study (Pearson's correlation coefficient $r = 0.69$, $p < 0.001$). Load changes determined by HC2 also precisely predicted histological progression of LSILs to HSILs.

In order to elucidate the causal relationship of integration in LSIL progression to HSILs using real-time PCR method, HPV 16, 18, 52, and 58 DNA samples were analyzed in 178 cervical swabs from women with LSILs and longitudinally monitored every 3 months in a multicenter trial. The integrated form was detected in only 5% (9/178) of samples with the episomal form. Additionally, no pure integration was found in women with LSILs due to HPV 16, 18, 52, and 58 at baseline and during follow-up. Furthermore, integration (both integrated DNA and episomal DNA) of HPV 16, 18, 52, or 58 DNA was detected in only 7.7% (1 of 13) of specimens of women during transition from LSILs to HSILs. Although some studies reported that early integration is noted in women with LSILs, the data suggests that integration is not often detected in women with LSILs, and probably is not essential for progression during transition from LSILs to HSILs (Kulmala et al, 2006). On the other hand, the prevalence of integration of HPV 16, 18, 52, or 58 DNA among women progressing from LSILs to HSILs is significantly increased ($p < 0.001$), which is comparable with previous studies showing that integration is often detected in HSILs (Peitsaro et al, 2004). Although, there was no difference for HPV 16 infection between women with LSILs and HSILs, this may be due to small sample size or reasons not yet known. The possible role of HPV DNA integration may be a late event in carcinogenesis, especially when it occurs in HSILs or cervical cancer, or when LSIL skips directly to HSIL after persistent HPV infection.

In conclusion, it is recommended that patients with LSIL and HPV types 16, 18, 52, or 58, consider detecting the viral load with real-time PCR or HC2 at the first visit and 6 months subsequently. Those with elevated viral loads are at risk of disease progression and should be carefully evaluated by colposcopy.

Although there is consensus that HPV integration is common in invasive cervical carcinomas and uncommon or absent in low-grade uterine cervical intraepithelial neoplasia (CIN I), estimates for HPV integration in CIN II/III range from 5% to 100% using different PCR-based and *in situ* hybridization (ISH) approaches. It has been suggested that HPV integration can be identified using ISH by scoring of punctate signals. The increased sensitivity of fluorescence ISH (FISH) methods, allowing the detection of single copies of HPV, complicates the distinction between integrated and episomal HPV. In reviewing the literature, Evans and Cooper commented on the wide range of estimates for integration in CIN II/III, i.e., 5% to 100% as determined by means of Southern blot

assays, different types of PCR analyses, as well as chromogenic and fluorescence *in situ* hybridization methods (FISH) (Lizard et al, 1994; Rihet et al, 1996, Ziol et al, 1998; Sano et al, 1998; Evans et al, 2002; Hopman et al, 2004). They concluded that, although questions remain regarding the prevalence of integration in preinvasive lesions, ISH approaches may be a viable alternative to PCR in determining both infections with high risk HPV as well as their physical status. The inherent problem of this approach is that signals originating from integrated HPV can be hidden in a background of episomal copies. Furthermore, HPV can also be present as concatamers, which makes the recognition of integrated HPV even more difficult (Evans and Cooper, 2004). Moreover, HPV DNA can contribute to the signals observed in these FISH protocols (Stoler and Broker, 1986; Kenny et al, 2002). In addition to integration, heavy viral loads in CIN lesions have recently been shown to increase the risk of carcinoma development *in situ* by at least 60-fold (Joseffson et al, 2000).

Although integration is proposed as a mechanism for progression from cervical intraepithelial neoplasia to carcinoma, it is unknown at what point this event occurs. Integration is commonly seen in invasive cervical carcinoma; however, a consensus has not been reached for high-grade lesions. More important may be the point at which transcriptional expression of these oncogenes pushes a cell toward malignancy. E6 mRNA has low or no transcription rate in latent/productive infection, and high transcription rate in persistent infection and malignant transformation. Positive mRNA detection at LSIL has a 69.8 times higher risk for development of HSIL; in contrast, positive HR HPV DNA detection has a 5.7 times higher risk (Molden et al, 2005). Previous studies demonstrated that better specificity of E6/E7 mRNA test (85%) versus HR HPV DNA test (50%) was achieved (Molden et al, 2005). RNA-based testing to detect E6/E7 mRNA transcripts may therefore be of higher prognostic value and improve the specificity of PPV compared to HPV DNA testing in screening. While DNA-based methods have been extensively evaluated in screening programs, the predictive values of mRNA-based methods are unknown. Few studies concerning the extent of oncogenic expression in cervical neoplasia have so far been published (Sotlar et al, 2004; Nakagawa et al, 2000; Lamarcq et al, 2002; Cuschieri et al, 2005; Kraus et al, 2004). Importantly, only a small proportion of HPV DNA–positive women with a normal, atypical squamous cell of uncertain significance or low-grade SIL diagnosis had a detectable mRNA expression. HPV E6/E7 mRNA detection by PreTect HPV-Proofer represents a promising new test as an adjunct to

cytology (Molden et al, 2005). HPV DNA testing will not discriminate between active and latent or transient infections. The mRNA test detects only 5 out of the 13 high-risk HPV types included in the DNA test. However, a recent meta-analyses have shown that these 5 high-risk HPV types (16, 18, 31, 33, and 45) are the predominant HPV types found in high-grade cervical neoplasia and comprise 97% of the oncogenic HPV types detected in cervical carcinomas in Europe and Northern America (Clifford et al, 2003). What percentage of mRNA transcripts from these 4 high-risk HPV types in Taiwan can be detected in the HPV DNA positive pre-invasive and SCC of the cervix is still unknown. Furthermore, the quantitation of mRNA level is probably correlated with the degree of cervical dysplasia (Scheurer et al, 2005).

Chapter 4: Conclusions and Perspective

Conclusions:

1. Real-time PCR detected HPV 16, 18 and 52 DNA in the peripheral blood of more than one-fourth of invasive cervical cancer patients. The association between risk of cancer recurrence and detection of viral DNA in blood among cervical cancer patients after treatment is suggestive and deserves further investigation.
2. Infrequent integration of HPV 52 and 58 DNA in cervical cancer suggests that it is not a prerequisite for progression to cervical cancer. High viral loads (E6) of HPV 16, 18 and 52 DNA may be predictive of the transition of CIN II-III to cervical cancer. These results indicate that both viral DNA physical status and viral loads of HPV are important factors in the carcinogenesis of different HPV types.
3. It is recommended that patients with LSIL, HPV types 16, 18, 52, or 58, consider detecting the viral load with real-time PCR or HC2 at the first visit and 6 months subsequently. Those with elevated viral loads are at risk of disease progression and should be carefully evaluated by colposcopy.

Perspective:

To continue this study and improve methods of predicting disease outcome, it is planned to develop a novel method of detecting integrated HPV DNA in smears by fluorescence *in situ* hybridization (FISH), and compare this with that detected by real-time PCR reported previously (Ho et al, 2005, 2006). In order to precisely predict the risk of cervical neoplastic progression, HPV DNA and mRNA detection methods will be compared in different stages - normal screening, pre-invasive and invasive cervical cancer - to observe whether HPV mRNA is more accurate in predicting cervical lesions.

Chapter 5: References

1. Alazawi W, Pett M, Strauss S, Moseley R, Gray J, Stanley M, Coleman N. Genomic imbalances in 70 snap-frozen cervical squamous intraepithelial lesions: associations with lesion grade, state of the HPV 16 E2 gene and clinical outcome. *Br J Cancer* 2004;91:2063-2070.
2. Anker P, Mulcahy H, Chen XQ, Stroun M. Detection of circulating tumor DNA in the blood (plasma/serum) of cancer patients. *Cancer Metastasis Rev* 1999;18:65–73.
3. Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesions Triage Study (ALTS) Group. Human papillomavirus testing for triage of women with cytologic evidence of low-grade squamous intraepithelial lesions: baseline data from a randomized trial. *J Natl Cancer Inst 2000*;92:397-402.
4. Badaracco G, Venuti A, Sedati A, Marcante ML. HPV 16 and HPV 18 in genital tumors: Significantly different levels of viral integration and correlation to tumor invasiveness. *J Med Virol* 2002;67:574-582.
5. Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J Virol.* 1987; 61:962–971.
6. Baseman JG, Koutsky LA. The epidemiology of human papillomavirus infections. *J Clin Virol* 2005;32(Suppl 1):S16-S24.
7. Bauer HM, Hildesheim A, Schiffman MH, Glass AG, Rush BB, Scott DR, Cadell DM, Kurman RJ, Manos MM. Determinants of genital human papillomavirus infection in low-risk women in Portland, Oregon. *Sex Transm Dis* 1993;20:274-278.
8. Bernard BA, Bailly C, Lenoir MC, Darmon M, Thierry F, Yaniv M. The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. *J Virol* 1989;63:4317-4324.
9. Beskow AH, Moberg M, Gyllensten UB. HLA class II allele control of HPV load in carcinoma in situ of the cervix uteri. *Int J Cancer* 2005;117:690-692.
10. Bhattacharjee B, Sengupta S. CpG methylation of HPV 16 LCR at E2 binding site proximal to P97 is associated with cervical cancer in presence of intact E2. *Virology* 2006;354:280-285.
11. Bosch FX, de Sanjose S. Chapter 1: Human papillomavirus and cervical

cancer - burden and assessment of causality. *J Natl Cancer Inst Monogr* 2003;31:3-13.

12. Boshart M, Gissman L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J* 1984;3:1151-1157.
13. Bremer GL, Tieboschb AT, van der Putten HW, de Haan J, Arends JW. P53 tumor suppressor gene protein expression in cervical cancer: relationship to prognosis. *Eur J Obstet Gynecol Reprod Biol* 1995;63:55-59.
14. Burd EM. Human papillomavirus and cervical cancer. *Clin Microbio Rev* 2003;16:1-17.
15. Capone RB, Pai SI, Koch WM, Gillison ML, Danish HN, Westra WH, Daniel R, Shah KV, Sidransky D.. Detection and quantitation of human papillomavirus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma. *Clin Cancer Res* 2000;6:4171–4175.
16. Castells A, Puig P, Mora J, Boadas J, Boix L, Urgell E, Sole M, Capella G, Lluis F, Fernandez-Cruz L, Navarro S, Farre A. K-*ras* mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. *J Clin Oncol* 1999;17:578–584.
17. Castle PE, Solomon D, Schiffman M, Wheeler CM, for the ALTS Group. Human papillomavirus type 16 infections and 2-year absolute risk of cervical precancer in women with equivocal or mild cytologic abnormalities. *J Natl Cancer Inst* 2005;97:1066-1071.
18. Center for Disease Control and Prevention. CDC National Prevention Information Network. Rockville, Md, 2004.
19. Chan SY, Bernard HU, Ong CK, Chan SP, Hofmann B, Delius H. Phylogenetic analysis of 48 papillomavirus types and 28 subtypes and variants: a showcase for the molecular evolution of DNA viruses. *J Virol* 1992;66:5714-5725.
20. Chen CA, Liu CY, Chou HH, Chou CY, Ho CM, Twu NF, Kan YY, Chuang MH, Chu TY, Hsieh CY. (2006) The distribution and differential risks of human papillomavirus genotypes in cervical preinvasive lesions: a Taiwan Cooperative Oncologic Group (TCOG) Study. *Int J Gynecol Cancer.* 2006;16(5):1801-1808.
21. Chen TM, Pecoraro G, Defendi V. Genetic analysis of *in vitro* progression of human papillomavirus-transfected human cervical cells. *Cancer Res* 1993;53:1167-1171.
22. Choo CK, Rorke EA, Eckert RL. Differentiation-independent constitutive expression

of the human papillomavirus type 16 E6 and E7 oncogenes in the CaSki cervical tumour cell line. J Gen Virol 1994;75:1139-1147.

23. Choo KB, Pan CC, Han SH. Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. *Virology* 1987;161:259-261.
24. Clifford GM, Smith JS, Aguado T and Franceschi S. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. *Br J Cancer* 2003;89:101–105.
25. Cuschieri KS, Cubie HA, Whitley MW, Gilkison G, Arends MJ, Graham C, McGoogan E. Persistent high risk HPV infection associated with development of cervical neoplasia in a prospective population study. *J Clin Pathol* 2005;58:946-950.
26. Dalstein V, Riethmuller D, Pretet JL, Carval KLB, Sautinere JL, Carbillet JP. Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. *Int J Cancer* 2003;106:396-403.
27. Daniel B, Rangarajan A, Mukherjee G, Vallikad E, Krishna S. The link between integration and expression of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions. *J. Gen. Virol* 1997;78**:**1095-1101.
28. Das BC, Sharma JK, Gopalarishna V, Luthra UK. Analysis by polymerase chain reaction of the physical state of human papillomavirus type 16 DNA in cervical preneoplastic and neoplastic lesions. *J Gen Virol* 1992;73:2327-2336.
29. Davies R, Hicks R, Crook T, Morris J, Vousden K. Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *J Virol* 1993;67:2521-2528.
30. Dong SM, Pai SI, Rha SH, Hildesheim A, Kurman RJ, Schwartz PE, Mortel R, McGowan L, Greenberg MD, Barnes WA, Sidransky D. Detection and quantitation of human papillomavirus DNA in the plasma of patients with cervical carcinoma. *Cancer Epidemiol Biomark Prev* 2002;11:3–6.
31. Dong XP, Stubenrauch F, Beyer-Finkler E, Pfister H. Prevalence of deletions of YY1-binding sites in episomal HPV 16 DNA from cervical cancers. *Int J Cancer* 1994;58:803-808.

32. Dowhanick JJ, McBride AA, Howley PM. Suppression of cellular proliferation by the papillomavirus E2 protein. *J Virol* 1995;69:7791-7799.
33. Duensing S, Lee LY, Duensing A, Basile J, Piboonniyom S, Gonzalez S Crum CP, Munger K. The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proc Natl Acad Sci USA* 2000;97:10002-10007.
34. Dürst M, Dzarlieva-Petrusevka T, Boukamp P, Fusenig NE, Gissmann L. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* 1987;1:251-252.
35. Dürst M, Glitz D, Schneider A, zur Hausen H. Human papillomavirus type 16 (HPV 16) gene expression and DNA replication in cervical neoplasia: analysis by *in situ* hybridization. *Virology* 1992;189:132-140.
36. Dyson N, Howley PM, Münger K, Harlow E. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989;243:934.
37. Evans MF, Mount SL, Beatty BG, Cooper K. Biotinyl-tyramide-based in situ hybridization signal patterns distinguish human papillomavirus type and grade of cervical intraepithelial neoplasia. *Mod Pathol* 2002;15:1339-1347.
38. Evans MF, Cooper K. Human papillomavirus integration: detection by in situ hybridization and potential clinical application. *J Pathol* 2004;202:1-4.
39. Finzer P, Soto U, Delius H, Patzelt A, Coy JF, Poustka A, zur Hausen H, Rosl F. Differential transcriptional regulation of the monocyte-chemoattractant protein-1 (MCP-1) gene in tumorigenic and non-tumorigenic HPV 18 positive cells: the role of the chromatin structure and AP-1 composition. *Oncogene* 2000;19:3235-3244.
40. Gravitt PE, Burk RD, Lorincz A, Herrero R, Hildesheim A, Sherman ME, Bratti MC, Rodriguez AC, Helzlsouer KJ, Schiffman M. A comparison between real-time polymerase chain reaction and hybrid capture 2 for human papillomavirus DNA quantitation. *Cancer Epidemiol Biomarkers Prev.* 2003;2:477-484.
41. Hampton GM, Penny LA, Baergen RN, Larson A, Brewer C, Liao S, et al. Loss of heterozygosity in cervical carcinoma: subchromosomal localization of a putative tumor-suppressor gene to chromosome 11q22-q24. *Proc Natl Acad Sci U S A* 1994;91:6953-6957.

42. Hildesheim A, Gravitt P, Schiffman MH, Kurman RJ, Barnes W, Jones S, Tchabo JG, Brinton LA, Copeland C, Epp J, Manos MM. Determinants of genital human papillomavirus infection in low-income women in Washington, D.C. *Sex Transm Dis* 1993;20:279-285.
43. Hopman AH, Smedts F, Dignef W, Ummelen M, Sonke G, Mravunac M, Vooijs GP, Speel EJ, Ramaekers FC. Transition of high-grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities. *J Pathol* 2004;202:23-33.
44. Huang J, Huang SL, Lin CY, Lin RW, Chao FY, Chen MY, Chang TC, Hsueh S, Hsu KH, Lai CH.. Human papillomavirus genotyping by a polymerase chain reaction-based genechip method in cervical carcinoma treated with neoadjuvant chemotherapy plus radical surgery. *Int J Gynecol Cancer* 2004;14:639-649.
45. Huang S, Afonina I, Miller BA, Bechmann M. Human papillomavirus types 52 and 58 are prevalent in cervical cancers from Chinese woman. *Int J Cancer* 1997;70:408–411.
46. Huibregtse JM, Scheffner M, Howley P. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol Cell Biol* 1993;13:775–784.
47. Huibregtse JM, Scheffner M, Howley PM. Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins, *Mol. Cell. Biol.* 1993;13:4918–4927.
48. IARC. Monographs on the evaluation of carcinogenic risks to humans: humanpapillomaviruses, Vol. 64. Lyon: International Agency for Research on Cancer; 1995.
49. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001;61:1659–1665.
50. Jen J, Wu L, Sidransky D. An overview on the isolation and analysis of circulating tumor DNA in plasma and serum. *Ann NY Acad Sci* 2000;906:8–12.
51. Jeon S, Lambert PF. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. *Proc Natl Acad Sci U S A* 1995;92:1654-1658.
52. Jeon SB, Allen-Hoffmann L, Lambert PF. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J*

Virol 1995;69:2989-2997.

53. Josefsson AM, Magnusson PK, Ylitalo N, Sorensen P, Qwarforth-Tubbin P, Andersen PK, Melbye M, Adami HO, Gyllensten UB. Viral load of human papillomavirus 16 and risk of cervical carcinoma *in situ*: a nested case-control study. *Lancet* 2000;355:2189–2193.
54. Keating JT, Ince T, Crum CP. Surrogate biomarkers of HPV infection in cervical neoplasia screening and diagnosis. *Adv Anat Pathol* 2001;8:83-92.
55. Kenny D, Shen LP, Kolberg JA. Detection of viral infection and gene expression in clinical tissue specimens using branched DNA (bDNA) in situ hybridization. *J Histochem Cytochem* 2002;50:1219-1227.
56. Klaes R, Woerner SM, Ridder R, Wentzensen N, Duerst M, Schneider A, Lotz B, Melsheimer P, von Knebel Doeberitz. Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. *Cancer Res* 1999;59:6132-6136.
57. Koi M, Morita H, Yamada H, Saboh H, Barrett JC, Oshimura M. Normal human chromosome 11 suppresses tumorigenicity of human cervical tumor cell line SiHa. *Mol Carcinog* 1989;2:12-21.
58. Kraus I, Molden T, Erno LE, Skomedal H, Karlsen F, Hagmar B. Human papillomavirus oncogenic expression in the dysplastic portio; an investigation of biopsies from 190 cervical cones. *Br. J. Cancer* 2004;90:1407–1413.
59. Kulmala SM, Syrjänen SM, Gyllensten UB, Shabalova IP, Petrovichev N, Tosi P, Syrjänen KJ, Johansson BC. Early integration of high copy HPV16 detectable in women with normal and low grade cervical cytology and histology. *Journal of Clinical Pathology* 2006;59:513-517.
60. Lamarcq L, Deeds J, Ginzinger D, Perry J, Padmanabha S and Smith-McCune K, Measurements of human papillomavirus transcripts by real time quantitative reverse transcription-polymerase chain reaction in samples collected for cervical cancer screening, *J. Mol. Diagnostics* 2002; 4:97–102.
61. Lambert APF, Anschau F, Schmitt VM. p16INK4A expression in cervical premalignant and malignant lesions. *Exp Mol Patho* 2006;80:192-196.
62. Lazcano-Ponce E, Herrero R, Muñoz N, Cruz A, Shah KV, Alonso P, Hernández P, Salmerón J, Hernández M. Epidemiology of HPV infection among Mexican women with normal cervical cytology. *Int J Cancer* 2001;91:412-420.
63. Lazo PA. Papillomavirus integration: prognostic marker in cervical cancer? Am. J.

Obstet. Gynecol 1997;176**:**1121-1122.

64. Lefevre J, Hankins C, Money D, Rachlis A, Pourreaux K, Coutlee F, Canadian Women's HIV Study Group. Human papillomavirus type 16 viral load is higher in human immunodeficiency virus-seropositive women with high-grade squamous intraepithelial lesions than in those with normal cytology smears. *J Clin Microbiol* 2004;42(5):2212-2215.
65. Ley C, Bauer HM, Reingold A, Schiffman MH, Chambers JC, Tashiro CJ, Manos MM. Determinants of genital human papillomavirus infection in young women. *J Natl Cancer Inst* 1991;83:997-1003.
66. Liaw KL, Hsing AW, Chen CJ, Schiffman MH, Zhang TY, Hsieh CY, Greer CE, You SL, Huang TW, Wu TC et al. Human papillomavirus and cervical neoplasia: a case-control study in Taiwan. *Int J Cancer* 1995;62:565–571.
67. Lieskovska J, Opavsky R, Zacikova L, Glasova M, Pastorek J, Pastorekova S. Study of in vitro conditions modulating expression of MN/CA IX protein in human cell lines derived from cervical carcinoma. *Neoplasma* 1999;46:17-24.
68. Liu VW, Tsang P, Yip A, Ng TY, Wong LC, Ngan HY. Low incidence of HPV DNA in sera of pretreatment cervical cancer patients. *Gynecol Oncol* 2001;82:269–272.
69. Lizard G, Chignol MC, Souchier C, Schmitt D, Chardonnet Y. Laser scanning confocal microscopy and quantitative microscopy with a charge coupled device camera improve detection of human papillomavirus DNA revealed by fluorescence in situ hybridization. *Histochemistry* 1994;101:303-310.
70. Lo YM, Chan LY, Chan AT, Leung SF, Lo KW, Zhang J, Lee JC, Hjelm NM, Johnson PJ, Huang DP. Quantitative and temporal correlation between circulating cell-free Epstein-Barr virus DNA and tumor recurrence in nasopharyngeal carcinoma. *Cancer Res* 1999;*59:*5452–5455.
71. Lorincz AT, Castle PE, Sherman ME, Scott DR, Glass AG, Wacholder S, Rush BB, Gravitt PE, Schussler JE, Schiffman M. Viral load of human papillomavirus and risk of CIN3 or cervical cancer.*Lancet* 2002;360:228-229.
72. Lungu O, Sun XW, Felix J, Richart RM, Silverstein S, Wright TC, Jr. Relationship of human papillomavirus type to grade of cervical intraepithelial neoplasia. *J Am Med Assoc* 1992;267:2493-2496.
73. Mantovani F, Banks L: The human papillomavirus E6 protein and its contribution to malignant progression. Oncogene 2001;20:7874-7887.
74. Meijer CJ, Snijders PJ, van den Brule AJ. Screening for cervical cancer: should we

test for infection with high-risk HPV? *CMAJ* 2000;163:535-538.

75. Melsheimer P, Vinokurova S, Wentzensen N, Bastert G, von Knebel, Doeberitz M. DNA aneuploidy and integration of human papillomavirus type 16 e6/e7 oncogenes in intraepithelial neoplasia and invasive squamous cell carcinoma of the cervix uteri. *Clin Cancer Res* 2004;10:3059.
76. Moberg M, Gustavsson I, Gyllensten U. Type-specific associations of human papillomavirus load with risk of developing cervical carcinoma in situ. *Int J Cancer* 2004; 112,854-859.
77. Moberg M, Gustavsson I, Wilander E, Gyllensten U. High viral loads of human papillomavirus predict risk of invasive cervical carcinoma. *Br J Cancer* 2005;92:891-894.
78. Molden T, Nygård JF, Kraus I, Karlsen F, Nygård M, Skare GB, Skomedal H, Thoresen S, Hagmar B. Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV-proofer and consensus PCR: A 2-year follow-up of women with ASCUS or LSIL Pap smear. Int J Cancer. 2005;114:973-976.
79. Münger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J Virol.* 1989;63(10): 4417–4421
80. Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M, Huh K. Mechanisms of human papillomavirus-induced oncogenesis. *J Virol* 2004;78:11451-11460.
81. Munger K, Basile JR, Duensing S, Eichten A, Gonzalez SL, Grace M, Zancy VL. Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. Oncogene 2001;20:7888-7898.
82. Munger K, Howley PM. Human papillomavirus immortalization and transformation functions. *Virus Res* 2002;89:213-228.
83. Muñoz N, Bosch FX, Castellsagué X, Díaz M, Sanjose Sd, Hammouda D,. Shah KV, Meijer CJ. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int J Cancer* 2004;111:278-285.
84. Muñoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ, International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003;348:518-527.
85. Murphy N, Ring M, Killalea AG, Uhlmann V, O'Donovan M, Mulcahy F, Turner M,

McGuinness E, Griffin M, Martin C, Sheils O, O'Leary JJ. $P16^{INK4A}$ as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. *J Clin Pathol* 2003;56:56-63.

86. Nagao S, Yoshinouchi M, Miyagi Y, Hongo A, Kodama J, Itoh S, Kudo T. Rapid and sensitive detection of physical status of human papillomavirus type 16 DNA by quantitative real-time PCR. *J Clin Microbiol* 2002;40:863-867.
87. Nakagawa S, Yoshikawa H, Yasugi T, Kimura M, Kawana K and Matsumoto K, Matsumoto K, Yamada M, Onda T, Taketani Y. Ubiquitous presence of E6 and E7 transcripts in human papillomavirus-positive cervical carcinomas regardless of its type, *J. Med. Virol.* 2000;62:251–258.
88. National Cancer Report of National Cancer Registration System, Department of National Health, Taiwan, 2003.
89. Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* 1996;2:1035–1037.
90. Park JS, Hwang ES, Lee CJ, Kim CJ, Rha JG, Kim SJ, Namkoong SE, Um Sj. Mutational and fuctional analysis of HPV-16 URR derived from Korean cervical neoplasia. *Gynecol Oncol* 1999;74:23-29.
91. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001;94:153–156.
92. Peitsaro P, Johansson B, Syrjanen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol* 2002;40:886-891.
93. Pett MR, Alazawi WO, Roberts I, Dowen S, Smith DI, Stanley MA, Coleman N. Acquisition of high-level chromosomal instability is associated with integration of human papillomavirus type 16 in cervical keratinocytes. *Cancer Res* 2004;64:1359-1368.
94. Pirami L, Giache V, Becciolini A. Analysis of HPV16, 18, 31, and 35 DNA in pre-invasive and invasive lesions of the uterine cervix. *J Clin Pathol* 1997;50:600-604.
95. Pornthanakasem W, Shotelersuk K, Termrungruangleert W, Noravud N, Niruthisard S, Mutirangura A. Human papillomavirus DNA in plasma of patients with cervical cancer. *BioMed Central Cancer* 2001;1:2.
96. Prétet JL, Dalstein V, Monnier-Benoit S, Delpeut S, Mougin C. High risk HPV load estimated by Hybrid Capture II® correlates with HPV16 load measured by

real-time PCR in cervical smears of HPV16-infected women. *J Clin Virol* 2004;31:140-147.

97. Renshaw AA, Mody DR, Lozano RL, Volk EE, Walsh MK, Davey DD, Birdsong GG. Detection of adenocarcinoma in situ of the cervix in Papanicolaou tests: comparison of diagnostic accuracy with other high-grade lesions. *Arch Pathol Lab Med* 2004;128:153-157.
98. Rihet S, Lorenzato M, Clavel C. Oncogenic human papillomaviruses and ploidy in cervical lesions. *J Clin Pathol* 1996;49:892-896.
99. Romanzuk H, Howley P. Disruption of either the E1 and E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. *Proc. Natl. Acad. Sci.* 1992;9**:**3159-3163.
100. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG, Sidransky D. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res* 2000;60:892–895.
101. Sano T, Hikino T, Niwa Y, Kashiwabara K, Oyama T, Fukuda T, Nakajima T. In situ hybridization with biotinylated tyramide amplification: detection of human papillomavirus DNA in cervical neoplastic lesions. *Mod Pathol* 1998;11:19-23.101.
102. Scheffner M, Werness B, Huibregtse J, Levine A, Howley P. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 1990;63:1129–1136.102.
103. Schiffman M, Hildesheim A, Herrero R, Bratti C.. HPV DNA testing in cervical cancer screening: results from women in a high-risk province of Costa Rica. *JAMA* 2000 283:87–93.
104. Scheurer ME, Tortolero-Luna G, Martial Guillaud M, Follen M, Chen Z, Laura M. Dillon LM and Karen Adler-Storthz K Correlation of Human Papillomavirus Type 16 and Human Papillomavirus Type 18 E7 Messenger RNA Levels with Degree of Cervical Dysplasia. Cancer *Epidemiology Biomarkers & Prevention* 2005; 14, 1948-1952103.
105. Schiller JT, Davies P. Delivering on the promise: HPV vaccines and cervical cancer. *Nat Rev Microbiol* 2004;2:343-347.
106. Seagon S, Durst M. Genetic analysis of an *in vitro* model system for human papillomavirus type 16-associated tumorigenesis. *Cancer Res* 1994;54:5593-5598.
107. Sherman ME, Schiffman M, Cox JT. Effects of age and human papilloma viral load on colposcopy triage: data from the randomized Atypical Squamous Cells of

Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study (ALTS). *J Natl Cancer Inst(Bethesda)* 2002;94:102-107.

108. Sherman ME, Lorincz AT, Scott DR, Wacholder S, Castle PE, Glass AG, Mielzynska-Lohnas I, Rush BB, Schiffman M. Baseline cytology, human papillomavirus testing, and risk for cervical neoplasia: a 10-year cohort analysis. J Natl Cancer Inst 2003; 95:46–52.
109. Sidransky D. Circulating DNA: what we know and what we need to learn. *Ann NY Acad Sci* 2000;906:1–4.
110. Silins I, Kallings I, Dillner J. Correlates of the spread of human papillomavirus infection. *Cancer Epidemiol Biomarkers Prev* 2000;9:953-959.
111. Snijders PJF, Steenbergen RDM Heideman DAM, Meijer CJLM. HPV-mediated cervical carcinogenesis: concepts and clinical implications. 2006;208:152-164.
112. Solomon D, Schiffman M, Tarone R. Comparison of three management strategies for patients with atypical squamous cells of undetermined significance: baseline results from a randomized trial. *J Natl Cancer Inst* 2001;93:293-299.
113. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright T, Jr, Young N;Forum Group Members; Bethesda 2001 Workshop. The 2001 Bethesda system: terminology for reporting results of cervical cytology. *JAMA* 2002;287:2114-2119.
114. Sotlar K, Stubner A, Diemer D, Menton S, Menton Mand Dietz K Wallwiener D, Kandolf R, Bültmann B., Detection of high-risk human papillomavirus E6 and E7 oncogene transcripts in cervical scrapes by nested RT-polymerase chain reaction, *J. Med. Virol* 2004; 74:107–116.
115. Soto U, Das BC, Lengert M, Finzer P, zur Hausen H, Rosl F. Conversion of HPV 18 positive non-tumorigenic HeLa-fibroblast hybrids to invasive growth involves loss of TNF-alpha mediated repression of viral transcription and modification of the AP-1 transcription complex. *Oncogene* 1999;18:3187-3198.
116. Steenbergen RD, Walboomers JM, Meijer CJ, van der Raaij-Helmer EM, Parker JN, Chow LT, Broker TR, Snijders PJ. Transition of human papillomavirus type 16 and 18 transfected human foreskin keratinocytes towards immortality: activation of telomerase and allele losses at 3p, 10p, 11q and/or 18q. *Oncogene* 1996;13:1249-1257.
117. Steenbergen RD, Kramer D, Braakhuis BJ, Stern PL, Verheijen RH, Meijer CJ, Snijders PJ. TSLC1 gene silencing in cervical cancer cell lines and cervical

neoplasia. *J Natl Cancer Inst* 2004;96:294-305.

118. Stoler MH, Broker TR. In situ hybridization detection of human papillomavirus DNAs and messenger RNAs in genital condylomas and a cervical carcinoma. *Hum Pathol* 1986;17:1250-1258.
119. Stoler MH, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT, Broker TR. Human papillomavirus type 16 and 18 gene expression in cervical neoplasia. *Hum Pathol* 1992;23:117.
120. Stroun M, Maurice P, Vasioukhin V, Lyautey J, Lederrey C, Lefort F, Rossier A, Chen XO, Anker P. The origin and mechanism of circulating DNA. *Ann NY Acad Sci* 2000;906:161–168.
121. Sun CA, Hsiung CA, Lai CH, Chen CA, Chou CY, Ho CM, Twu NF, Feng WL, Chuang MH, Hsieh CY, Chu TY. Epidemiologic correlates of cervical human papillomavirus prevalence in women with abnormal pap smear tests: a Taiwan Cooperative Oncology Group (TCOG) Study. *J Med Virol* 2005;77:273-281.
122. Syrjänen K, Syrjänen S. Papillomavirus infections in human pathology. J. Wiley & Sons, Inc., New York, N.Y., 2000
123. "10-county HPV screening study" - Total number = 10,000 Specimen: self-obtained swabs, Department of Health, Taiwan, 2005. (unpublished data).
124. Thomas JT, Oh ST, Terhune SS, Laimins LA. Cellular changes induced by low-risk human papillomavirus type 11 in keratinocytes that stably maintain viral episomes. *J. Virol.*2001;75**:**7564-7571.
125. Tjalma WAA Role of human papillomavirus in the carcinogenesis of squamous cell carcinoma and adenocarcinoma of the cervix J Best Practice & Research Clinical Obstetrics & Gynaecology 2005;19:469-483.
126. Tseng CJ, Pao CC, Lin JD, Soong YK, Hong JH, Hsueh S. Detection of human papillomavirus types 16 and 18 mRNA in peripheral blood of advanced cervical cancer patients and its association with prognosis. *J Clin Oncol.* 1999;17:1391-1396.
127. Van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA, van den Brule AJ,. Verheijen RH, Helmerhorst TJ, Meijer CJ Human papillomavirus 16 load in normal and abnormal cervical scrapes: An indicator of CIN II/III and viral clearance. *Int J Cancer* 2002;98(4): 590 – 595.
128. Vernon SD, Unger ER, Miller DL, Lee DR, Reeves WC. Association of human papillomavirus type 16 integration in the E2 gene with poor disease-free survival

from cervical cancer. *Int J Cancer* 1997;74:50-56.

129. Von Knebel Doeberitz M, Rittmuller C, Aengeneyndt F, Jansen-Durr P, and Spitkovsky D. Reversible repression of papillomavirus oncogene expression in cervical carcinoma cells: consequences for the phenotype and E6-p53 and E7-pRB interactions *J. Virol.*1994; 68:2811-2821.
130. Walboomers JMM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N.Human papillomavirus is a necessary cause of invasive cancer worldwide. *J Pathol* 1999;189:12–19.
131. Wallin KL, Wiklund F, Angstrom T, Bergman F, Stendahl U, Wadell G, Hallmans G, Dillner J. Type-specific persistence of human papillomavirus DNA before the development of invasive cervical cancer. *N Engl J Med* 1999;341:1633-1638.
132. Wentzensen N, Vinokurova S, von Knebel DM: Systemic review of genomic integration sites of human papillomavirus genomes in epithelial dysplasia and invasive cancer of the female lower genital tract. Cancer Res 2004;64:3878-3884.
133. Widschwendter A, Blassnig A, Wiedemair A, Muller-Holzner E, Muller HM, Marth C. Human papillomavirus DNA in sera of cervical cancer patients as tumor marker. *Cancer Lett* 2003;202:231–239.
134. Wilke CM, Hall BK, Hoge A, Paradee W, Smith DI and Glover TW. FRA3B extends over a broad region and contains a spontaneous HPV16 integration site: direct evidence for the coincidence of viral integration sites and fragile sites. *Hum. Mol. Genet.*1996;5,187–195.
135. Williams GH, Romanowski P, Morris L, Madine M, Mills AD, Stoeber K, Marr J, Laskey RA, Coleman N. Improved cervical smear assesment using antibodies against proteins that regulate DNA replication. Proc Natl Acad Sci USA 1998;95:14932-14937.
136. Winer RL, Kiviat NB, Hughes JP, Adam DE, Lee SK, Kuypers JM, Koutsky LA. Development and duration of human papillomavirus lesions, after initial infection. *J Infect Dis* 2005;191:731.
137. Ylitalo N, Sorensen P, Josefsson AM, Magnusson PK, Andersen PK, Ponten J, Adami HO, Gyllensten UB, Melbye M. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma *in situ*: a nested case-control study. *Lancet* 2000;355:2194–2198.
138. Yokoyama M, Nakao Y, Yang XL, Qi Sun, Tsutsumi K, Pater A, Pater MM. Alterations in physical state and expression of human papillomavirus type 18 DNA

following crisis and establishment of immortalized ectocervical cells. Virus Research 1995;37:*139-151.*

139. Zhao W, Noya F, Chen WY, Townes TM, Chow LT, Broker TR. Trichostatin A. up-regulates human papillomavirus type 11 upstream regulatory region-E6 promoter activity in undifferentiated primary human keratinocytes. *J Virol* 1999;73:5026-5033.
140. Zielinski GD, Snijders PJ, Rozendaal L, Voorhorst FJ, van der Linden HC, Runsink AP, de Schipper FA, Meijer CJ. HPV presence precedes abnormal cytology in women developing cervical cancer and signals false negative smears. *Br J Cancer* 2001;85:398-404.
141. Zielinski GD, Snijders PJ, Rozendaal L, Daalmeijer NF, Risse EK, Voorhorst FJ, Jiwa NM, van der Linden HC, de Schipper FA, Runsink AP, Meijer CJ. The presence of high-risk HPV combined with specific p53 and p16INK4a expression patterns points to high-risk HPV as the main causative agent for adenocarcinoma *in situ* and adenocarcinoma of the cervix. *J Pathol* 2003;201:535-543.
142. Ziol M, Di Tomaso C, Biaggi A, Tepper M, Piquet P, Carbillon L, Uzan M, Guettier C. Virological and biological characteristics of cervical intraepithelial neoplasia grade I with marked koilocytotic atypia. *Hum Pathol* 1998;29:1068-1073.
143. zur Hausen H. Papillomavirus infections—a major cause of human cancers. *Biochim. Biophys. Acta.* 1996 1288**:**F55-F78.
144. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002;2(5):342-350.

Chapter 6: List of Tables

Table 1. HPV detector primer set

	Primer set	Amplicon size
Type16	MY74 5' CAT TTG TTG GGG TAA CCA AC3' MY75 5' TAG GTC TGC AGA AAA CTT TTC 3'	412 bps
Type 18	MY76 5' TGT TTG CTG GCA TAA TCA AT 3' MY77 5' TAA GTC TAA AGA AAA CTT TTC 3'	415 bps
Type 52	MY81F 5' CAC TTC TAC TGC TAT AAC TTG T 3' MY82R 5' CCT TTC CTT TAG GTG TGT 3'	50 bps
HLA-DQα	GH26 5' GTG CTG CAG GTG TAA ACT TGT ACC AG 3' GH27 5' CAC GGA TCC GGT AGC GGT AGA GTT G3'	242 bps

Table 2. Detection of HPV-16, -18 or 52 DNA by Real - Time Quantitative PCR

Cases with HPV-positive blood	Age	HPV typing		HPV concentration		Disease stage and site	SCC	Tumor size (cm)
		Blood	Swab	Before	After treatment			
1	63	16	16	1.035	ND	IB	0.3	2.0
2	53	16	16	111.5	1756	R, bone metastasis	0.8	
3	69	16	16	1.83	ND	R, abdominal metastasis	0.4	3.0
		18		ND	<1 7.94			
4	48	18	18	<1	ND	IB	0.2	4.2
5	43	18	ND	<1	<1	IB, R in abdomen	6.7	4.5
6	56	18	18	<1	ND	R, pelvic metastasis, NED after treatment	0.8	8.0
7	51	18	16	<1	5.07, 0.32	IIB	2.2	5.0
8	78	18	18	<1	ND	IB	1.8	3.0
9	53	18	ND	1.17	<1	R, bone metastasis Adeno CA		
10	36	18	18	<1	2.64	IB, R in pelvis	1.9	3.1
11	56	18	18	6.0	ND	IB	5.6	3.0
12	57	52	52	689, 3700,	584, 92, ND	IIB, LN metastasis	4.0	6.0
13	56	52	16, 52	1.34	38	IIIB, R in neck LN metastasis	5.8	7.0
14	47	52	52	8.2	ND	IB Adeno CA	3.0	
15	68	52	52	69	ND	IIB, persistent, abdominal metastasis	5.3	5.0
		18	18	ND	<1			
16	84	52	52	17.8	77.75	IVB, persistent	4.5	5.5

ND: not detectable R: recurrence NED: no evidence of disease LN: lymph node

Table 3. Primer sequences of E2 and E6 in HPV type 16, 18, 52 and 58.

Oligo Name	Sequence (5'→3')	Site in HPV Genome
16E2-F	AATTATTAGGCAGCACTTGGCCA	3381-3403
16E2-R	ATCTTGGTCGCTGGATAGTCGTCT	3481-3458
16E6-F	GAGCGACCCAGAAAGTTACCAC	122-243
16E6-R	ACCTCACGTCGCAGTAACTGTTG	228-206
18E2-F	CCGCTACTCAGCTTGTTAAACAGCT	3454-3478
18E2-R	GCCGACGTCTGGCCGTAG	3550-3533
18E6-F	CGGCGACCCTACAAGCTACC	129-148
18E6-R	ACCTCTGTAAGTTCCAATACTGTCTTGC	235-208
52E2-F	ACTGAAACTGCTGTCCACCTATGC	3361-3384
52E2-R	TGACGTCTGGTCGTCGTCG	3454-3436
52E6-F	ACACGACCCCGGACCCT	120-136
52E6-R	CTTGTATACCTCTCTTCGTTGTAGCTCTTT	233-204
58E2-F	CCACTACTGAAACTGCTGACCCAA	3366-3389
58E2-R	GGGTGTTGTCTCTGGAGTCTGGTAA	3473-3449
58E6-F	GGAGAAACCACGGACATTGCA	127-147
58E6-R	ACCTCAGATCGCTGCAAAGTC	234-214

Table 4. Clinical characteristics and median E6 load for LSIL of 61 eligible women with low-grade squamous intraepithelial lesions (LSILs) due to human papillomavirus (HPV) 16, 18, 52, or 58 with at least four follow-up visits

	HPV 16	**HPV 18**	**HPV 52**	**HPV 58**
Percentage of each type	23.0% (14/61)	13.1% (8/61)	42.6% (26/61)	21.3% (13/61)
Median age (range)	35 (range: 24–58)	31 (range: 23–51)	41 (range: 24–65)	38 (range: 20–52)
Median E6 load for LSIL	13437	26599	35620	37366
Range of viral load for LSIL	0–735527	0–5029143	0–3843486	0–2754113
Median E6 load for HSIL	31916	21067	156906	110107
Range of viral load for HSIL	0–34386322	0–41748	0–13596437	0–14243132
P value (median E6 load between LSIL and HSIL)	0.049	0.499	0.032	0.184

HSILs = high-grade squamous intraepithelial lesions

Table 5. Odds ratios (OR) and 95% confidence intervals (95% CI) for 2-year cumulative high-grade squamous intraepithelial lesion (HSIL) diagnoses associated with human papillomavirus (HPV) 16, 18, 52, and 58 status, viral load change, age, high viral load and repeat Pap smear.

Characteristic	OR (95% CI)	p-value
HPV Risk		
HPV 16 (n = 14)	1.5 (0.4–5.8)	0.34
HPV 18 (n = 8)	0.6 (0.1–5.3)	0.23
HPV 52 (n = 26)	0.7 (0.2–2.6)	0.34
HPV 58 (n = 13)	0.3 (0.0–2.1)	1.61
Baseline viral load		
HPV DNA > 10^5/ or HPV DNA ≤l 10^5	0.8 (0.2–3.1)	0.07
Viral load change		
Viral load increase between baseline and 6 month follow-up for HPV testing		
Real-time PCR	8.3 (2.0–34)	< 0.01
Hybrid capture 2	5.6 (1.5–21.4)	< 0.01
HPV Persistence		
Persistent HPV/Transient	2.5 (0.5–12.7)	0.27
Age at enrollment		
≥ 30 or <30	1.8 (0.3–11.7)	0.19
Repeat Pap smear		
Abnormal or normal	2.1 (0.5–8.3)	1.27

p-value < 0.05 represents statistical significance (chi square).

List of Figures

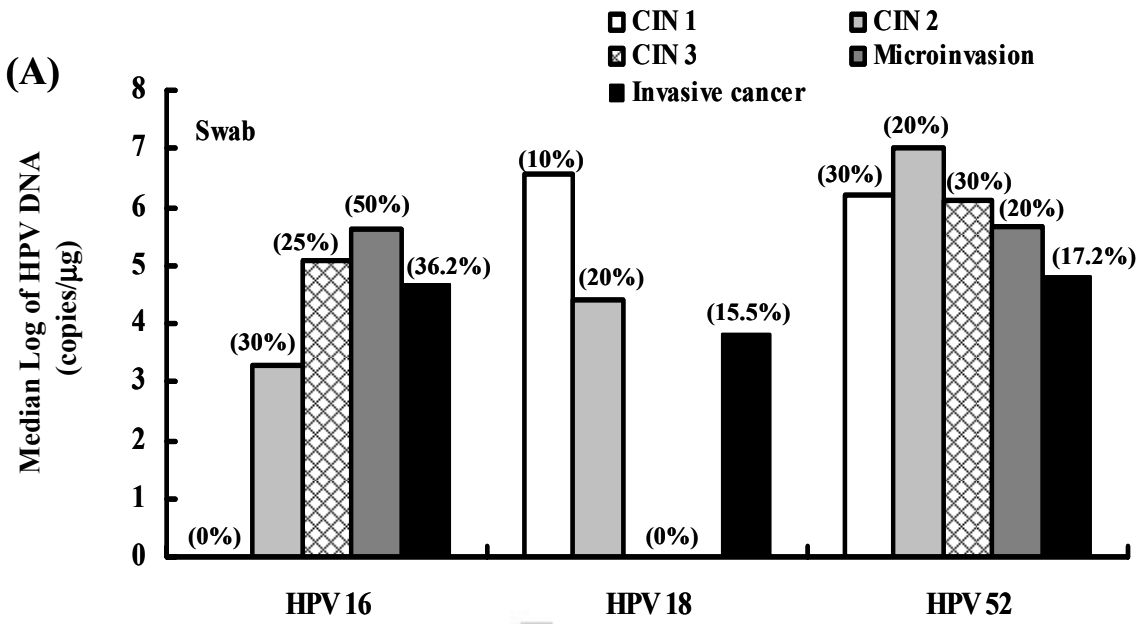

Figure 1(A). The dynamic change in concentration (copies/genome) of HPV DNA type 16, 18 and 52 detected in cervical swabs indicated as copies/genome. The amount of viral DNA was detected by real-time PCR using a sub-type specific primer. The viral load in blood and the amount of viral DNA in cervical swab samples were plotted with each type of HPV and for different stages of cervical cancer (CIN I, CIN II, CIN III, microinvasion and invasive cancer). The prevalence of each diagnosis is indicated at the top of each column.

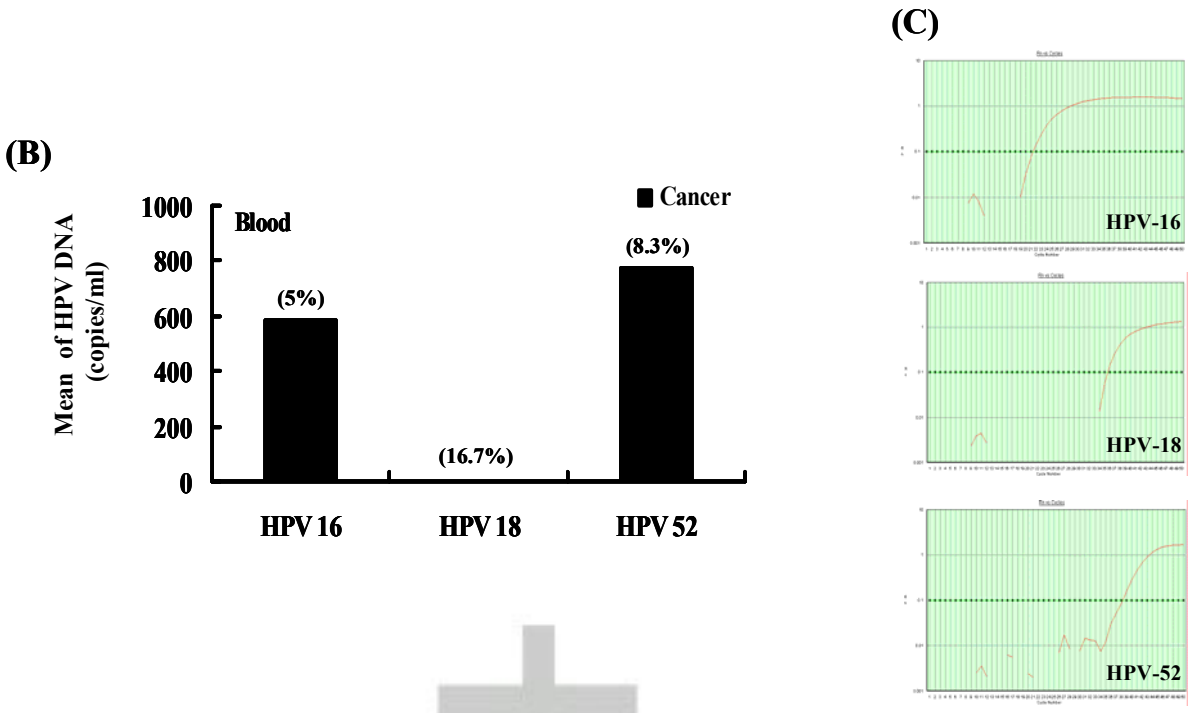

Figure 1(B). The dynamic change in concentration (copies/genome) of HPV DNA type 16, 18 and 52 detected in blood specimens indicated as copies/ml.

Figure 1(C). A representative amplification curve for each type of HPV DNA from a patient sample.

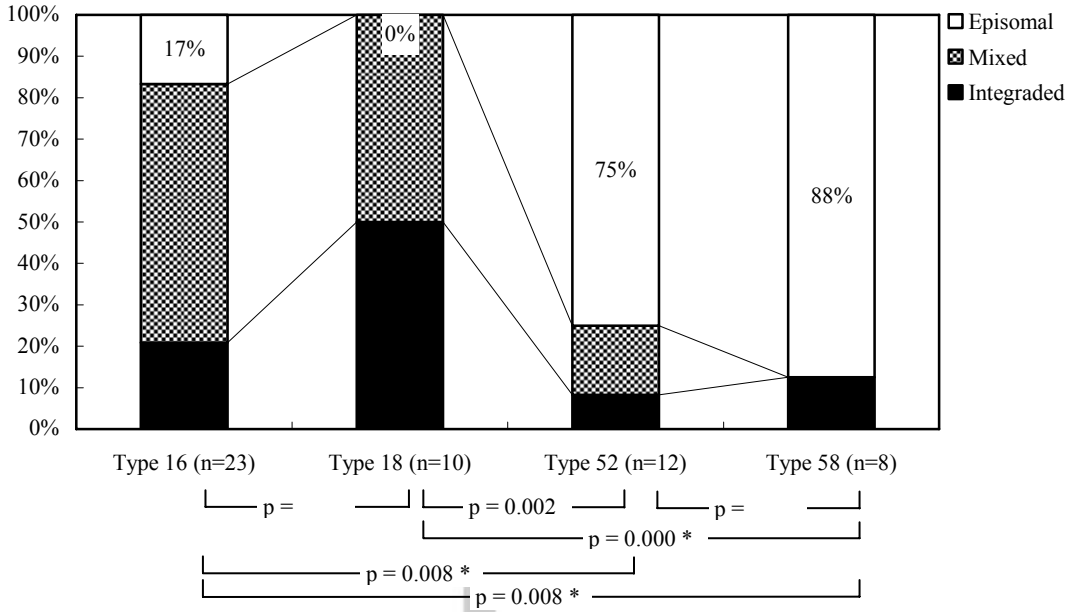

Figure 2. Differences in the prevalence of the physical status of HPV 16, 18, 52 and 58 DNA detected in the swabs of cervical cancer patients. The segments (white, stippled, and black) represent ratios of specimens harboring HPV 16, 18, 52 and 58 in pure episomal, mixed, and pure integrated forms, respectively. $p<0.05$ represents a significant difference.

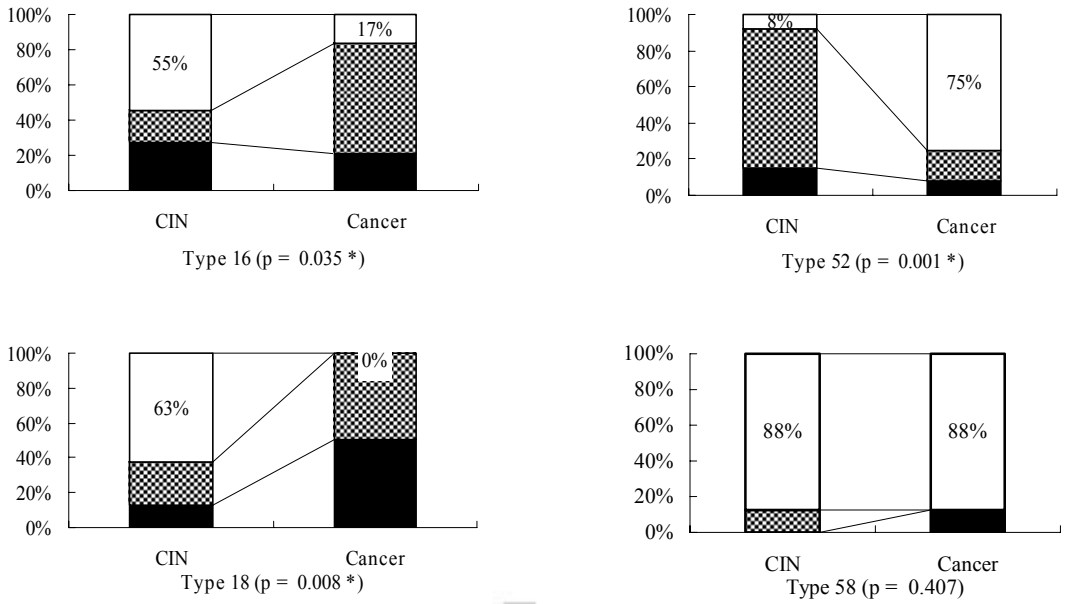

Figure 3. Differences in the prevalence of physical status of HPV 16, 18, 52 and 58 DNA between swabs of patients with cervical cancer and CIN II-III. The segments (white, stippled, and black) represent ratios of specimens harboring HPV 16, 18, 52 and 58 in pure episomal, mixed, and pure integrated forms, respectively. $p<0.05$ represents a significant difference.

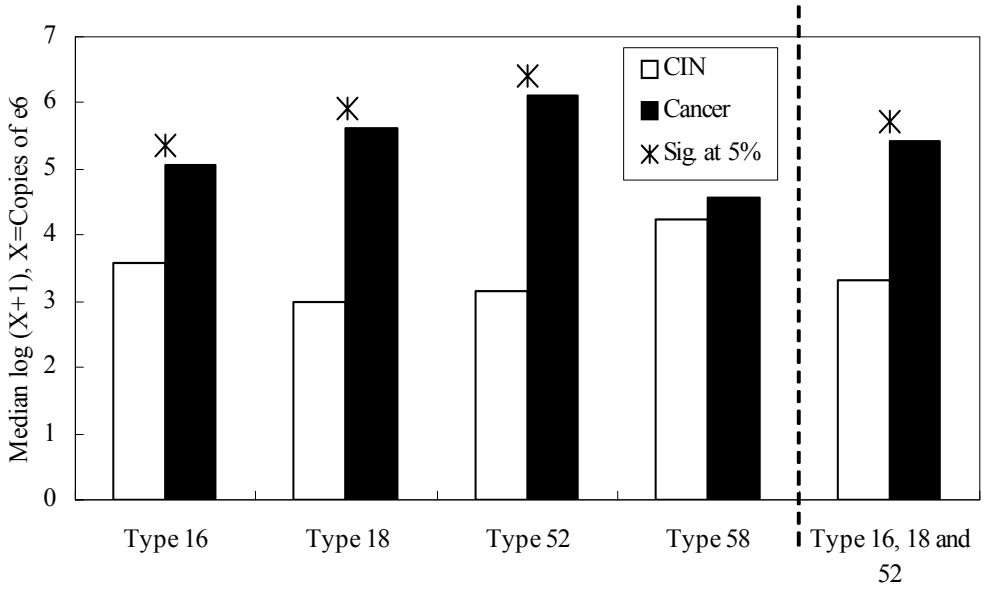

Figure 4. Differences in the median log of E6 viral loads of HPV DNA type 16, 18, 52 and pooled data between CIN II-III and cervical cancer. $p<0.05$ represents significant.

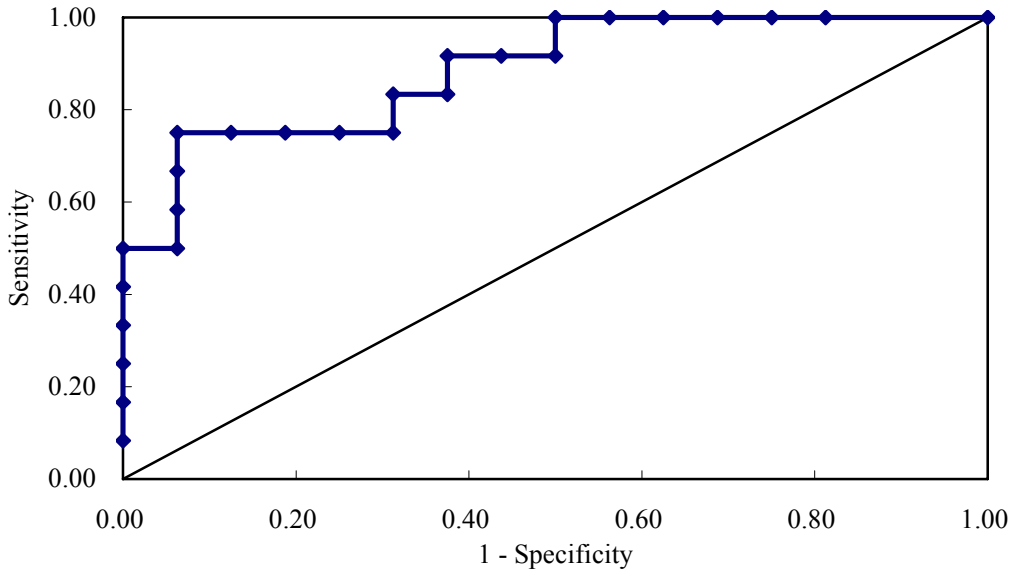

Figure 5. Prediction of CIN II-III or cervical cancer using the ROC curve of the median log of E6 viral loads of HPV 52 DNA.

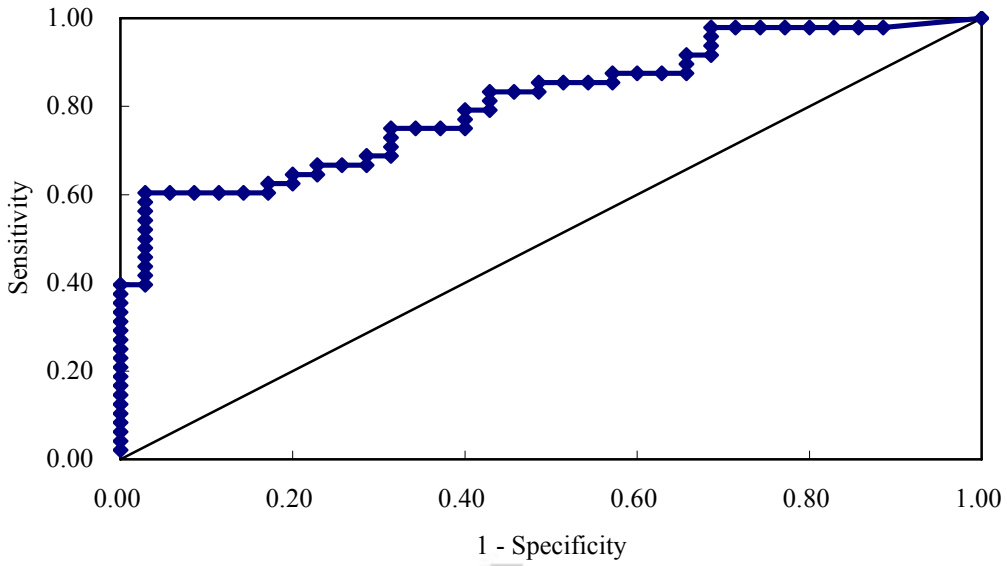

Figure 6. Prediction of CIN II-III or cervical cancer using the ROC curve of the median log of E6 viral loads of pooled data of HPV 16, 18 and 52 DNA.

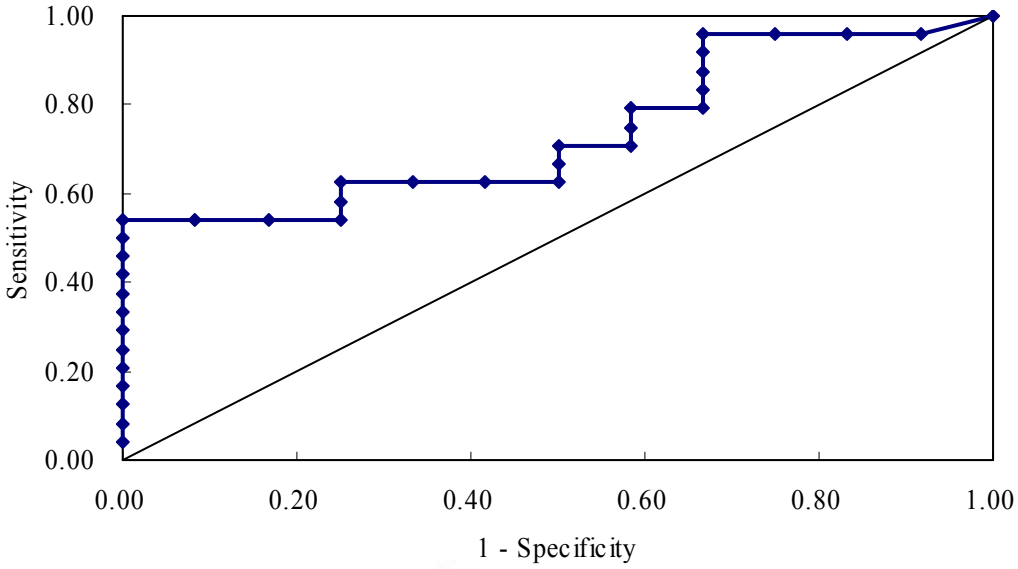

Figure 7. Prediction of CIN II-III or cervical cancer using the ROC curve of the median log of E6 viral loads of HPV 16 DNA.

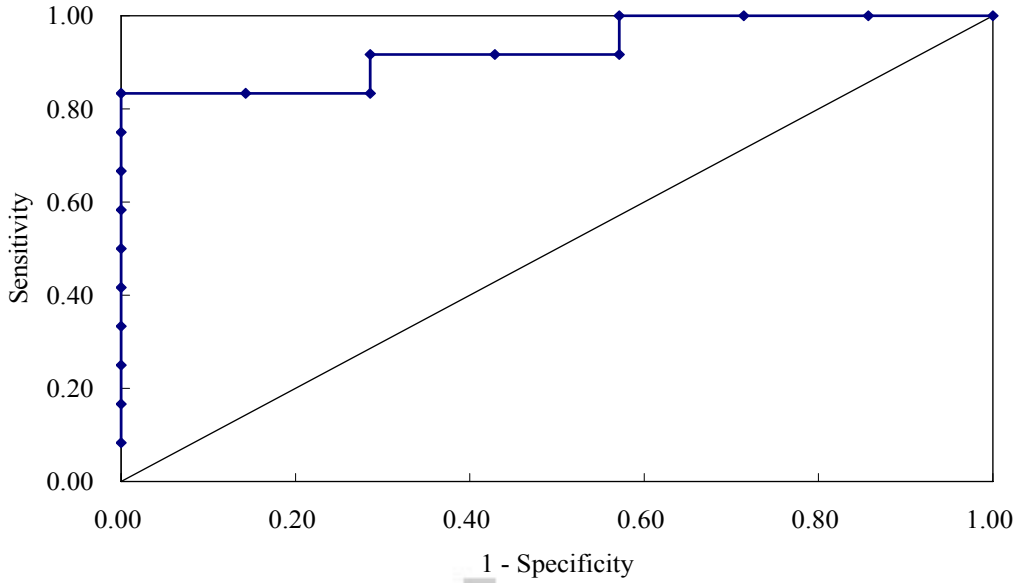

Figure 8. Prediction of CIN II-III or cervical cancer using the ROC curve of the median log of the E6 viral load of HPV 18 DNA.

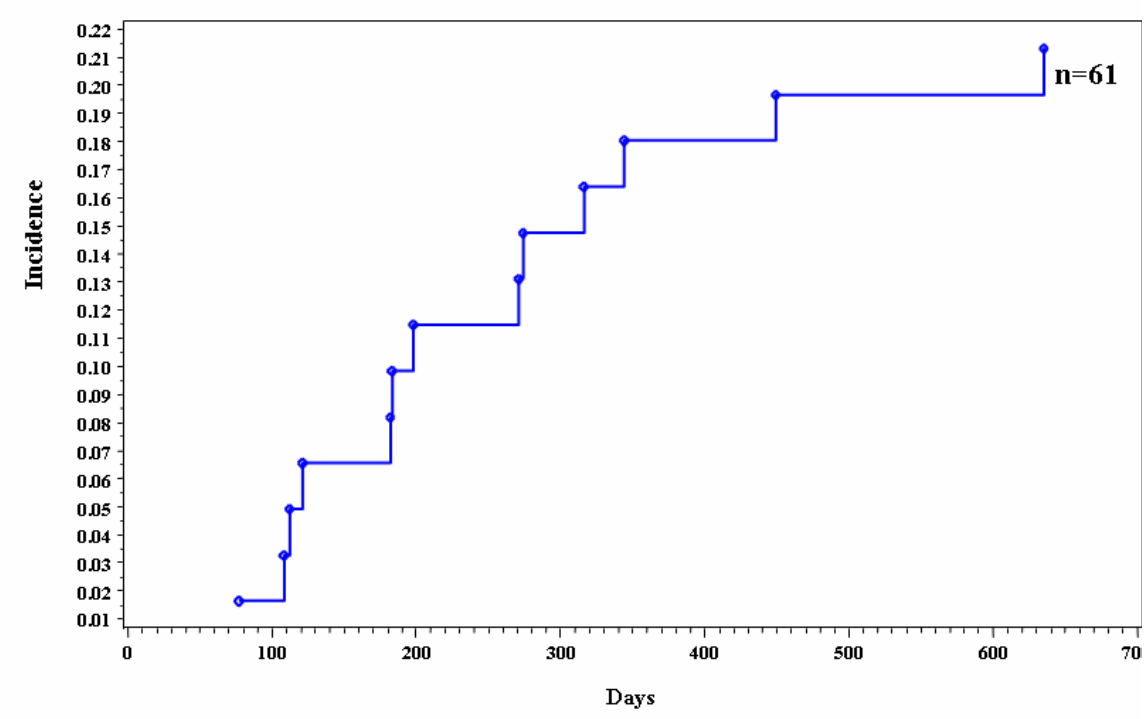

Figure 9. Kaplan-Meier estimates of cumulative incidence of high-grade squamous intraepithelial lesions (HSILs) among the 61 women with low-grade squamous intraepithelial lesions (LSILs), according to HPV types 16, 18, 52 and, or 58.

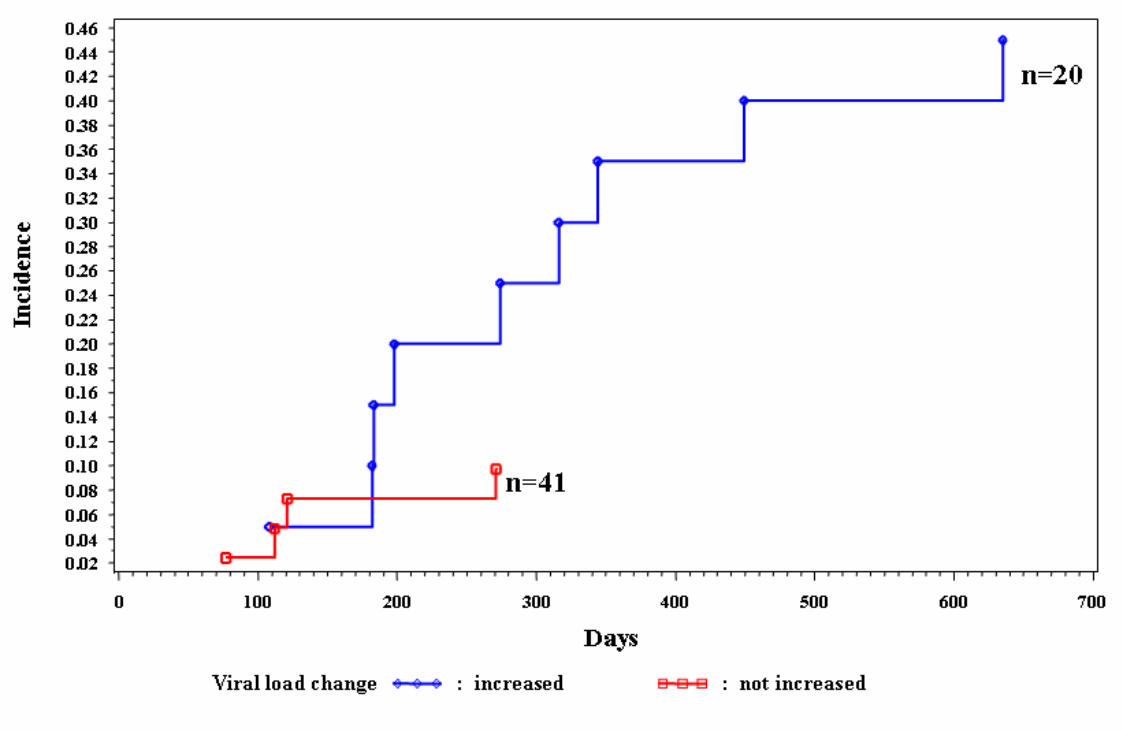

Figure 10.Kaplan-Meier estimates of cumulative incidence of high-grade squamous intraepithelial lesions (HSILs) among the 61 women with low-grade squamous intraepithelial lesions (LSILs), according to viral load change of HPV for HPV 16, 18, 52, and 58 (load increased versus not increased).

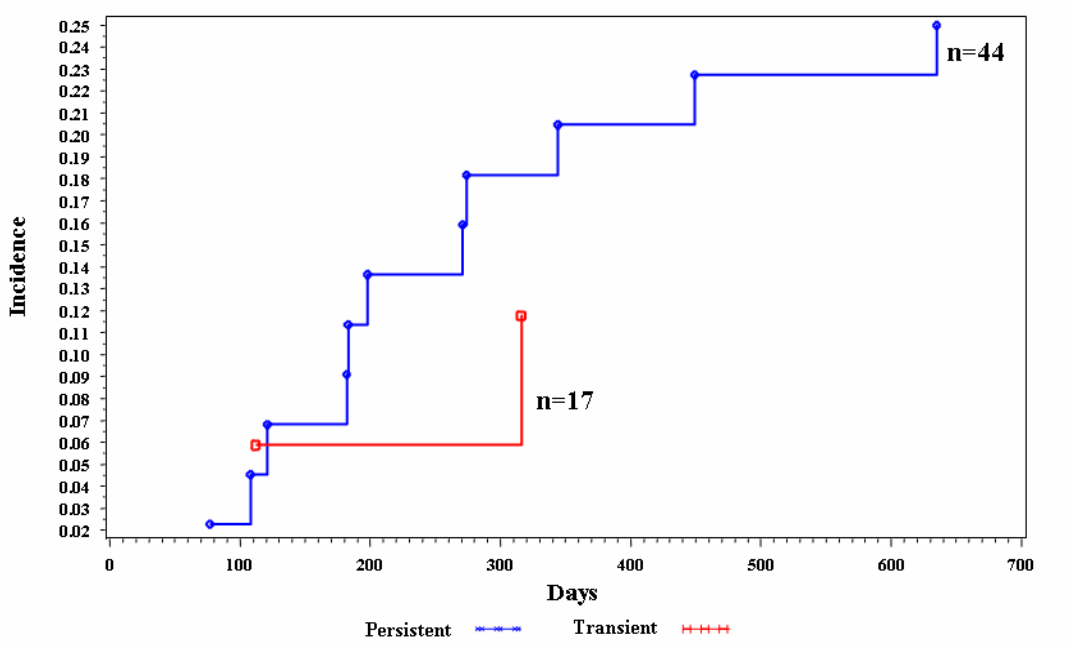

Figure 11. Kaplan-Meier estimates of cumulative incidence of high-grade squamous intraepithelial lesions (HSILs) among the 61 women with low-grade squamous intraepithelial lesions (LSILs), according to transient versus persistent infection of HPV with HPV 16, 18, 52 and, or 58.