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# Association between Quantitative High-Risk Human Papillomavirus DNA Load and Cervical Intraepithelial Neoplasm Risk

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## Abstract

Human papillomavirus (HPV) infection is a high-risk factor for cervical intraepithelial neoplasm (CIN) but the association between the quantitative HPV DNA load and the severity of CIN remains controversial. We conducted a community study to investigate the correlation between the two. Potential study subjects were selected through Pap smear screening in Kaohsiung County, Taiwan. Ninety-one subjects with either their first case of inflammation or  $\geq$ CIN1 by biopsy confirmation were assigned to a case group; 175 normal subjects with negative findings by Pap smears or biopsies were assigned to a control group. Cervical HPV load was detected with Hybrid Capture II assay for high-risk HPV infection, with nested PCR for high- and low-risk HPV infection, and with type-specific PCR for HPV type 16 (HPV-16). Individuals with positive high-risk HPV infection had an

increased risk of developing CIN. Compared with HPV-negative subjects, the odds ratios were 32.2 [95% confidence interval (95% CI), 10.4-99.5] for subjects with CIN1, 37.2 (95% CI, 7.4-187.6) for subjects with CIN2, and 68.3 (95% CI, 14.1-328.5) for subjects with  $\geq$ CIN3 after adjusting for other confounding factors. The similar trend was also found among the HPV-16-negative individuals. In addition, high-risk HPV DNA load levels were highly correlated with the different grades of CINs in the overall population (Spearman's correlation coefficient  $r = 0.67$ ,  $P < 0.0001$ ,  $n = 266$ ) and the HPV-16-negative population (Spearman's correlation coefficient  $r = 0.58$ ,  $P < 0.0001$ ,  $n = 234$ ). We concluded that high-risk HPV infection, irrespective of HPV-16 infection, was highly and positively associated with the development of CIN. (Cancer Epidemiol Biomarkers Prev 2005;14(11):2544-9)

## Introduction

Without treatment, high-grade cervical intraepithelial neoplasm (CIN2-3) can develop into invasive cervical carcinoma (1). High-risk human papillomavirus (HPV) infection (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), a major risk factor of cervical cancer (2, 3), accounts for 43% to 53% of CIN subjects in the western population (4, 5). Like the cytologic test, although the HPV test is used as a screening tool for cervical neoplasm (5-9), the relationship between the quantitative titer of the HPV DNA load and the histologic severity of cervical lesions is still unclear (5, 10-13). Some studies have found the different levels of high-risk HPV DNA to correlate with the grade of CIN (5, 10, 11). Others have reported only a high HPV-16 load, not other high-risk HPV infections, to be positively associated with the severity of cervical lesions (12, 13). In this study, we continue to explore the relationship between HPV infection and CIN severity. To avoid inconsistencies found in previous studies, we used three different methods of detecting HPV: Hybrid Capture II assay for high-risk HPV infection, nested PCR for high- and low-risk HPV infection, and type-specific PCR for HPV-16.

## Materials and Methods

**Study Area.** Kaohsiung County, located on the southwestern coast of Taiwan, has an area of  $\sim 2,792.7$  km<sup>2</sup> and 27 administrative districts. Of the 27 districts, we chose to study 15 districts ( $\sim 537.6$  km<sup>2</sup>), those which have an average population density more than or equal to the average population density (623 persons/km<sup>2</sup>) of Taiwan.

**Participants.** This is an ongoing community-based nested case-control study. We cooperated with the Kaohsiung County Bureau of Health administrative staff members who provided the surveillance information about Pap smear screening. In total, 145,616 women, ages  $\geq 20$  years, underwent Pap smear screening in these 15 districts between January 2003 and September 2004. Of these 145,616 women, 1,377 were diagnosed for the first time as having a lesion equal to or greater than cervical intraepithelium neoplasm I ( $\geq$ CIN1). After excluding those who were undergoing direct therapy (786), those who we were unable to reach (390), and those who were uncooperative or disqualified for other reasons (64), we were left with 137 women who were willing to participate in our study for interview and HPV tests and were asked to have biopsy information. Another 43 of 137 subjects with abnormal Pap smears did not perform the cervical biopsies later for unknown reasons. However, the mean age ( $\pm$ SD) was 46.2 years ( $\pm 13.2$ ) for subjects with biopsies ( $n = 94$ ) and 43.6 years ( $\pm 10.8$ ) for those without biopsies ( $n = 43$ ) with no statistically significant differences ( $t$  statistics =  $-1.12$ , degrees of freedom = 135,  $P = 0.26$ ).

The cervical biopsies were done in a variety of hospitals decided by study subjects and the reporting pathologists in this study did not know their statuses of HPV infection.

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Because Pap smear screening was operated by the Taiwan government, it was mandatory for pathologists to report the final biopsy diagnoses for the women who were screened at Kaohsiung County Bureau of Health.

Potential controls were randomly selected from women whose areas of residence were within the same administrative districts as the study cases and whose past and present Pap smear reports were all negative. Our goal was a case-control ratio of 1:1-2 age matched to within 1 year. In total, we recruited 172 eligible controls. This study was approved by Kaohsiung Medical University Institutional Review Board. After being selected, all eligible cases and controls were asked to sign informed consent forms, provide Pap smears collected by trained public health nurses, and fill out a standardized questionnaire.

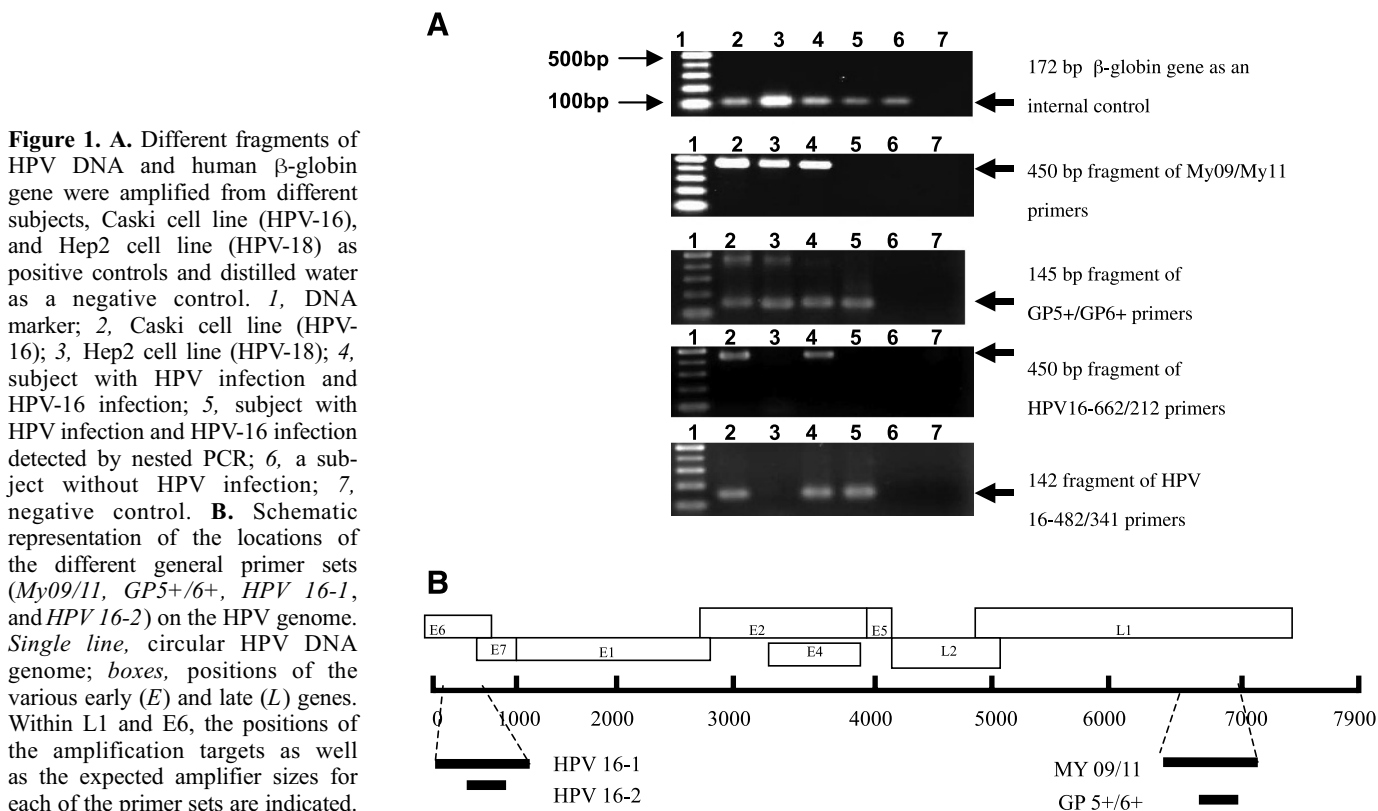
**Collection of Cervical Specimens.** Specimens were taken from each study participant's cervix by a trained public health nurse using a Cytobrush (DIGENE, Gaithersburg, MD) and cervical swab. The Cytobrush was immersed in 1-mL specimen transport medium and the cervical swab in 5-mL PBS solution. Both were swirled to release the cells. The technicians examining the specimens for HPV infection in this study were blinded to the findings of Pap smears and cervical biopsies.

**HPV DNA Detected Using Hybrid Capture II Assay.** The Cytobrush specimens in the specimen transport medium were initially stored at 4°C until the HPV analysis and then analyzed using a commercial kit for performing Hybrid Capture II method (DIGENE) according to the instructions of the manufacturer. This ELISA is based on a sandwich hybridization followed by a nonradioactive alkaline phosphatase reaction with chemiluminescence in microplates. The hybrid complex of high-risk HPV, including subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, can be detected by the DIGENE DML 2000 Luminometer. The results were analyzed by DIGENE DML 2000 Software and the Pentium II PC System. Light measurements were expressed as relative light units (RLU). A solution with 10 pg/mL of high-risk HPV

served as positive control. The ratio of a specimen's RLU to the corresponding positive control's RLU was considered a measurement of viral load. In addition, according to the instructions of the manufacturer, an RLU ratio of  $\geq 1.0$  in a specimen was a positive indication of the presence of HPV DNA whereas a ratio of  $< 1.0$  was a negative indication (14).

**DNA Extraction from Cotton Stick Solution.** Using the phenol-chloroform extraction method, we extracted DNA from the PBS solution in which the cervical swab specimen was immersed. The PBS solution was first centrifuged at 1,500 rpm for 15 minutes at room temperature. The supernatant was then discarded. Five-hundred microliters of cell lysis buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L EDTA) were added and the solution was incubated at room temperature for 5 minutes. Five-hundred microliters of phenol-chloroform were then added to the water phase and the mixture was centrifuged to remove the water phase. Finally, the DNA was collected by ethanol precipitation and the pellet was washed, dried, and dissolved in double-distilled water (15).

**Nested PCR for High- and Low-Risk HPV Infection.** Approximately 100 ng of genomic DNA from each sample were initially amplified using  $\beta$ -globin primers as an internal control (172 bp) in a 25- $\mu$ L reaction mixture (Fig. 1A). The human  $\beta$ -globin gene was successfully amplified in all of the (100%) cervical swab specimens. The amplified samples were then used to amplify HPV DNA. The condition for nested PCR for high- and low-risk HPV infection was the reaction mixture (50  $\mu$ L) containing 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl<sub>2</sub>, 10  $\mu$ g gelatin, 250  $\mu$ mol/L deoxynucleotide triphosphate, 1.25 units of *Thermus aquaticus* (Taq) DNA polymerase (16), and primers (each 30 pmol; outer primer pairs in the first-step PCR and inner primer pairs in the second-step PCR; ref. 17). The outer primers (My09 and My11) were CGTCCMARRGAWACTGATC and GCMCAGGWCA-TAAYAATGG (M = A + C, R = A + G, W = A + T, and Y = C + T; ref. 8); the inner primer pairs (GP5+ and GP6+) were



**Figure 1. A.** Different fragments of HPV DNA and human  $\beta$ -globin gene were amplified from different subjects, Caski cell line (HPV-16), and Hep2 cell line (HPV-18) as positive controls and distilled water as a negative control. 1, DNA marker; 2, Caski cell line (HPV-16); 3, Hep2 cell line (HPV-18); 4, subject with HPV infection and HPV-16 infection; 5, subject with HPV infection and HPV-16 infection detected by nested PCR; 6, a subject without HPV infection; 7, negative control. **B.** Schematic representation of the locations of the different general primer sets (My09/11, GP5+/6+, HPV 16-1, and HPV 16-2) on the HPV genome. Single line, circular HPV DNA genome; boxes, positions of the various early (E) and late (L) genes. Within L1 and E6, the positions of the amplification targets as well as the expected amplifier sizes for each of the primer sets are indicated.

TTTGTTACTGTGGTAGATACTAC and GAAAAATAAACTGTAAATCATATTC (Fig. 1B; ref. 7). PCR was done under a denaturation condition of 95°C for 3 minutes, followed by 35 cycles at 94°C for 15 seconds, 42°C for 2 minutes, and 72°C for 20 seconds, and a final extension at 72°C for 7 minutes. All PCR products were electrophoresed on a 3% agarose gel and visualized on an UV transilluminator after ethidium bromide staining. The sizes of PCR products for outer (My09 and My11) and inner (GP5+ and GP6+) primers were ~450 and ~145 bp, respectively (Fig. 1A; refs. 7, 8).

For quality control, we added one positive control of 100 pg purified HPV DNA from the Caski cell line (HPV-16) and one negative control from distilled water in each set of runs (~10 samples). The HPV subtypes 6, 11, 13, 16, 18, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, and 66 can be detected through this nested PCR method (17).

**Type-Specific PCR for HPV-16 Infection.** After the presence of HPV infection was detected by GP5+ and GP6+ primers using the above nested PCR method, presence of specific HPV-16 subtype was then determined based on the following nested PCR method. The condition for type-specific HPV-16 infection nested PCR was a reaction mixture (50  $\mu$ L) containing 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl<sub>2</sub>, 10  $\mu$ g gelatin, 250  $\mu$ mol/L deoxynucleotide triphosphate, 1.25 units of Taq DNA polymerase (16), and primers (each 30 pmol; outer primer pairs in the first-step PCR and inner primer pairs in the second-step PCR; ref. 17). Initially, the specific outer primers (HPV16-662: TCCCTGAGCTGTCATTTAATTGC and HPV16-212: TTACTGCGACGTGAGGTATATGACT) were used to amplify a fragment of ~450 bp in the E6 region of HPV-16. Then, the inner primers (HPV16-482: TGATTA-CAGCTGGGTTTCTCTACGT and HPV16-341: TCAAAGC-CACTGTGTCCTGAA) were used in the second-step PCR to generate a fragment of ~142 bp (Fig. 1; ref. 17). The PCR condition was done under a denaturation condition of 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

**Statistical Analyses.** The distribution of demographic characteristics in different grades of CIN was analyzed by Fisher's exact test because the small sample size was present in some categories of variables. The odds ratios (OR) with their

95% confidence intervals (95% CI) of the association between HPV infection and CIN risk were estimated by logistic regression models after controlling for other covariates. The different distributions of HPV DNA load in different grades of CIN were analyzed by one-way ANOVA and Student's *t* statistics after normalizing RLU levels by a natural logarithm transformation. The correlations between RLU levels and the grades of CIN were examined by Spearman's rank correlation. The data were analyzed on SAS statistical software.

## Results

Among the 94 subjects with abnormal Pap smear reports and completing the biopsy follow-up, 3 had normal findings, 32 had inflammations, and 26 had CIN1, 12 CIN2, and 21  $\geq$ CIN3. The 172 subjects with normal Pap smear reports and the 3 subjects with abnormal Pap smear reports but normal cervical biopsy reports were combined for later analysis. Significant differences were found between various distributions of smoking status, prior Pap smears, and number of lifetime sexual partners among the designated categories of normal, inflammation, CIN1, CIN2, and  $\geq$ CIN3. Family history of cervical cancer was marginally significant. There were no other statistically significant differences found in demographics (Table 1).

Table 2 shows the correlation between the severity of CIN and HPV infection as measured by different methods. The positive rate of Hybrid Capture II method increased from 11% in the subjects with normal results and 72% in the subjects with inflammation to 81%, 84%, and 90% in the subjects with CIN1, CIN2, and  $\geq$ CIN3, respectively. Using normal subjects as a baseline, we found the ORs to be 19.3 (95% CI, 7.6-48.9) for subjects with inflammation, 32.2 (95% CI, 10.4-99.5) for subjects with CIN1, 37.2 (95% CI, 7.4-187.6) for subjects with CIN2, and 68.3 (95% CI, 14.1-328.5) for subjects with  $\geq$ CIN3 after adjusting for smoking status, number of prior Pap smears, number of lifetime sexual partners, and family history of cervical cancer. Similar results were found using the nested PCR method for high- and low-risk HPV infection using the primers of My09/My11 and GP5+/GP6+.

As expected, the positive rate of specific HPV-16 infection was highly correlated with the severity of CIN (Table 2). However, even after excluding subjects with positive report of

**Table 1. Distributions of demographic characteristics and other potential confounders (n = 266)**

Variables	Normal, n = 175	Inflammation, n = 32	CIN1, n = 26	CIN2, n = 12	$\geq$ CIN3, n = 21	P*
Age (y)						
<46	84 (48%)	19 (60%)	17 (66%)	4 (33%)	10 (47%)	0.26
$\geq$ 46	91 (52%)	13 (40%)	9 (34%)	8 (67%)	11 (53%)	
Education levels						
$\geq$ Junior high school	114 (65%)	20 (63%)	18 (70%)	7 (59%)	8 (38%)	0.17
$\leq$ Primary school	61 (35%)	12 (37%)	8 (30%)	5 (41%)	13 (62%)	
Smoking status						
Nonsmoker	65 (37%)	7 (22%)	3 (12%)	1 (8%)	3 (14%)	0.001
ETS exposure <sup>†</sup>	104 (59%)	21 (66%)	19 (73%)	10 (84%)	14 (67%)	
Smoker	6 (3%)	4 (12%)	4 (15%)	1 (8%)	4 (19%)	
No. of prior Pap smears						
<2	11 (6%)	6 (19%)	5 (19%)	2 (17%)	6 (29%)	0.002
$\geq$ 2	164 (94%)	26 (81%)	21 (81%)	10 (83%)	15 (71%)	
Number of lifetime sexual partners						
$\leq$ 1	161 (92%)	27 (84%)	19 (73%)	8 (67%)	19 (90%)	0.007
>1	14 (8%)	5 (16%)	7 (27%)	4 (33%)	2 (10%)	
Age at first intercourse (y)						
>22	99 (57%)	17 (53%)	16 (62%)	6 (50%)	6 (29%)	0.15
$\leq$ 22	76 (43%)	15 (47%)	10 (38%)	6 (50%)	15 (71%)	
Family history of cervical cancer						
Yes	6 (3%)	3 (9%)	0 (0%)	1 (9%)	3 (14%)	0.06
No	169 (97%)	29 (91%)	26 (100%)	11 (91%)	18 (86%)	

\*Fisher's exact test.

<sup>†</sup>ETS, environmental tobacco smoke.

**Table 2. Adjusted ORs and 95% CIs of CIN associated with HPV infection**

Variables	Normal	Inflammation	CIN1	CIN2	≥CIN3
High-risk HPV infection detected by Hybrid Capture II method ( <i>n</i> = 266)					
Positive	19 (11%)	23 (72%)	21 (81%)	10 (84%)	19 (90%)
Negative	156 (89%)	9 (28%)	5 (19%)	2 (16%)	2 (10%)
OR (95% CI)	1.0	20.9 (8.4-51.9)	34.5 (11.6-102)	41.1 (8.3-201)	78.0 (16.8-361)
Adjusted OR (95% CI)*	1.0	19.3 (7.6-48.9)	32.2 (10.4-99.5)	37.2 (7.4-187.6)	68.3 (14.1-328.5)
Nested PCR for high- and low-risk HPV infection ( <i>n</i> = 266)					
Positive	29 (17%)	26 (81%)	21 (81%)	11 (92%)	20 (95%)
Negative	146 (83%)	6 (19%)	5 (19%)	1 (8%)	1 (5%)
OR (95% CI)	1.0	21.8 (8.2-57.7)	21.1 (7.3-60.6)	55.3 (6.8-445.7)	100.6 (12.9-780.2)
Adjusted OR (95% CI)*	1.0	20.1 (7.4-54.4)	17.5 (5.9-51.6)	46.1 (5.6-377.8)	93.5 (11.5-755.2)
HPV-16 infection ( <i>n</i> = 266)					
Positive	5 (3%)	4 (12%)	8 (31%)	5 (42%)	10 (48%)
Negative	170 (97%)	28 (88%)	18 (69%)	7 (58%)	11 (52%)
OR (95% CI)	1.0	4.8 (1.2-19.1)	15.1 (4.5-51.1)	24.3 (5.7-103.7)	30.9 (8.9-106.3)
Adjusted OR (95% CI)*	1.0	4.5 (1.1-18.7)	17.5 (4.7-64.0)	26.2 (5.6-120.2)	28.5 (7.6-105.6)
High-risk HPV infection detected by Hybrid Capture II method without HPV-16 infection ( <i>n</i> = 234)					
Positive	18 (11%)	20 (71%)	13 (72%)	5 (71%)	10 (91%)
Negative	152 (89%)	8 (29%)	5 (28%)	2 (29%)	1 (9%)
OR (95% CI)	1.0	21.1 (8.1-54.8)	21.9 (7.0-68.7)	21.1 (3.8-116.8)	84.4 (10.2-698.5)
Adjusted OR (95% CI)*	1.0	18.9 (7.1-50.8)	19.6 (5.9-65.5)	19.2 (3.2-111.8)	78.6 (9.2-674.4)

\*Adjusting for smoking status, number of prior Pap smears, number of lifetime sexual partners, and family history of cervical cancer.

specific HPV-16 infection, we still found the positive rate for other high-risk HPV infections, detected by the Hybrid Capture II method, to be highly and positively associated with the severity of CIN. Compared with normal subjects, the ORs were 18.9 (95% CI, 7.1-50.8) for subjects with inflammations, 19.6 (95% CI, 5.9-65.5) for subjects with CIN1, 19.2 (95% CI, 3.2-111.8) for subjects with CIN2, and 78.6 (95% CI, 9.2-674.4) for subjects with ≥CIN3 after adjusting for other covariates (Table 2).

The high-risk HPV DNA load levels (mean ± SE) were found to increase gradually starting from normal subjects to CIN2 (normal, 1.7 ± 0.8; inflammation, 165.1 ± 50.9; CIN1, 418.2 ± 151.6; CIN2, 805.6 ± 219.3) and then started decreasing in ≥CIN3 (334.5 ± 105.8) subjects (Table 3). This tendency was also found in HPV-16–negative subjects (Table 3). High-risk HPV DNA load levels, when based on the RLU ratio detected by the Hybrid Capture II method, were highly correlated with the severity of CIN (Spearman's correlation coefficient  $r = 0.67$ ,  $P < 0.0001$ ,  $n = 266$ ; Fig. 2). The results remained similar even when we excluded those with positive HPV-16 reports (Spearman's correlation coefficient  $r = 0.58$ ,  $P < 0.0001$ ,  $n = 234$ ).

## Discussion

This study finds high-risk HPV infection to be highly and positively associated with the severity of CIN regardless of HPV-16 infection involvement. In addition, high-risk HPV DNA load levels, measured by RLU ratio detected by the Hybrid Capture II method, correlated significantly with the severity of CIN although the HPV DNA load was lower in the CIN3 group than in the CIN1 and CIN2 groups.

Previous studies on the relationship between quantitative levels of HPV DNA and histologic severity of cervical lesions have yielded controversial results. Some investigators, using Hybrid Capture I or Hybrid Capture II, have found greater amounts of HPV DNA able to more precisely predict the progression of cervical lesion and HPV DNA load able to predict CIN grade. The more the HPV loads, the higher the CIN grade (5, 10, 11). Recently, Sherman et al. (18) found HPV DNA load to be slightly decreased in groups with CIN3. In their study, single and multiple types of HPV DNA levels (mean ± SE) were 440.1 ± 24.8 and 583.8 ± 33.0 in the ≤CIN1, 446.8 ± 59.5 and 620.5 ± 66.3 in the CIN2, and 313.2 ± 59.4 and 615.9 ± 63.1 in the ≥CIN3 groups, suggesting single or multiple HPV types can affect the HPV DNA load through the Hybrid Capture II method. In addition, they found the number of abnormal cells in the sample of Pap smear is also an important determinant of RLU level of Hybrid Capture II (18). In this study, we found the mean ± SE of the HPV load in those with ≥CIN3 (RLU, 334.5 ± 105.8) to be lower than in those with CIN1 (RLU, 418.2 ± 151.6) and CIN2 (RLU, 805.6 ± 219.3). This finding maybe explained by (a) small sample sizes in the group of ≥CIN3 in this study and that (b) less matured dysplastic squamous cells in CIN3, compared with CIN1 or CIN2, contained less viral DNA per cell (18-20).

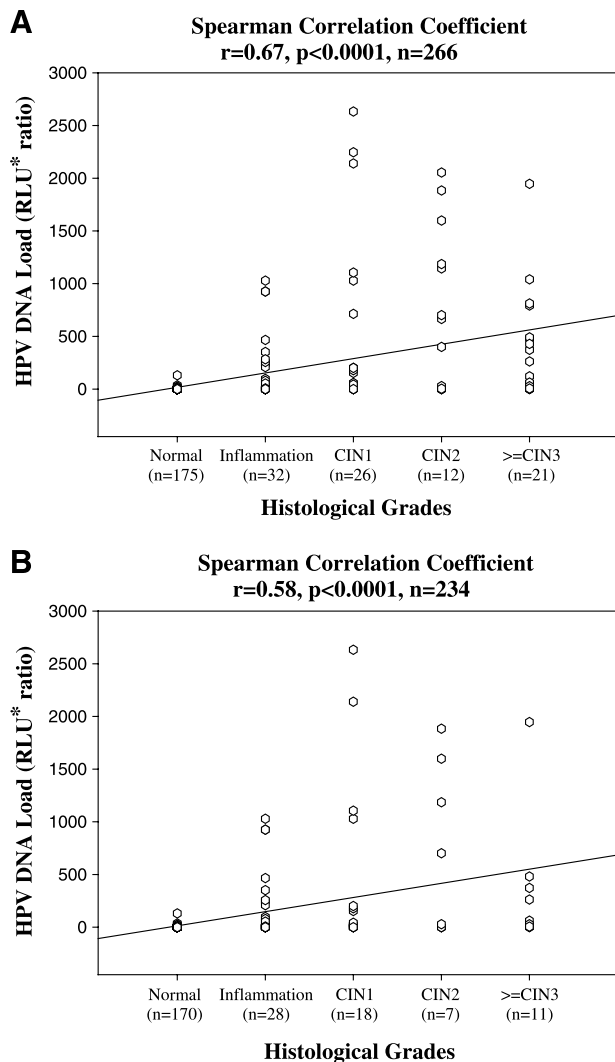
HPV-16 DNA load has been highly associated with the development of CIN (12, 13). Zerbini et al. (12) studied 176 subjects (atypical squamous cells of undetermined significance, 44; low-grade squamous intraepithelial lesion, 43; high-grade squamous intraepithelial lesion, 89) to examine the DNA load in specific high-risk HPV types 16, 18, 31, 33, and 45 as well as in low-risk HPV types 6 and 11 in a variety of cervical lesions using PCR-ELISA. They found very high titers

**Table 3. Relationship of natural log–transformed HPV DNA load level with CIN risk**

HPV DNA Load	Normal	Inflammation	CIN1	CIN2	≥CIN3
RLU ratio in overall subjects ( <i>n</i> = 266)					
<i>N</i>	175	32	26	12	21
Mean ± SE	1.7 ± 0.8	165.1 ± 50.9*	418.2 ± 151.6*	805.6 ± 219.3*	334.5 ± 105.8*
Min, med, max	0.1, 0.3, 130.8	0.2, 6.7, 1,029.3	0.2, 34.3, 2,632.7	0.3, 683.5, 2,053.7	0.5, 62.5, 1,946.6
RLU ratio in HPV-16–negative subjects ( <i>n</i> = 234)					
<i>N</i>	170	28	18	7	11
Mean ± SE	1.8 ± 0.8	168 ± 57.6*	420.4 ± 187.1*	771.4 ± 302.3 <sup>†</sup>	289.5 ± 173.5*
Min, med, max	0.1, 0.3, 130.8	0.2, 6.2, 1,029.3	0.2, 25.3, 2,632.7	0.3, 701.7, 1,883.3	0.8, 27.9, 1,946.6

\* $P < 0.001$ , in comparison with normal subjects.

<sup>†</sup> $P = 0.01$ , in comparison with normal subjects.



**Figure 2.** Correlation between HPV DNA load and cervical histologic grades detected by Hybrid Capture II method. **A.** Overall subjects. **B.** HPV-16–negative subjects.

of HPV-16 DNA (>1,000 genome copies per cell), not of other high-risk HPV types, to be positively correlated with the severity of cervical lesions. Ylitalo et al. (13), using a sensitive quantitative PCR to estimate HPV-16 load in multiple smears for each woman, found cervical carcinoma to be associated with HPV-16–positive women who were consistently found to have a high viral load over the long term. They found that cases with high viral loads would have an increased risk of up to 22.7% (95% CI, 12.4–31.8) of developing CIN. Other investigators found the prevalence rate of HPV-16 to be ~48% among the HPV-positive women in Taiwan (1, 21, 22). Our data showed a HPV-16 prevalence rate of 29.9% among nested PCR positive subjects and a HPV-16 prevalence rate of 39.0% among subjects with cervical lesion  $\geq$ CIN1, confirmed by biopsy.

The positive rate of specific HPV-16 infection was highly correlated with the severity of CIN. We detected HPV infection using Hybrid Capture II and nested PCR as well as type-specific HPV-16 infection. After adjusting for other potential confounders, we found positive high-risk HPV–infected women to be at higher risk of developing CIN than HPV-negative individuals in both the nested PCR and Hybrid Capture II methods. Our results were consistent even after excluding the HPV-16–positive population results. Therefore, it is likely that overall HPV infection can predict the development of CIN.

Although other investigations have found high-risk HPV DNA load to have a high correlation with CIN progression, they have not made clear the role HPV-16 in the relationship (5, 10, 11). In our study, we investigated the contribution of the high-risk HPV DNA load in both HPV-16–positive and HPV-16–negative individuals. Results consistently indicated that high-risk HPV DNA load correlated highly with development of CIN.

Although the sample size in this ongoing study was small, we found high-risk HPV infection, irrespective of HPV-16 infection, to be highly and positively associated with the severity of CIN. The women in this study were chosen from the Pap smear screening network of Kaohsiung County Bureau of Health. The findings of cervical biopsies were reported to Kaohsiung County Bureau of Health by different pathologists, which may introduce interobserver variability in the interpretation of biopsy specimens. The pathologists were blinded to the statuses of HPV infection of the women in this study, which could result in random misclassification of outcome in this study. In addition, the lab staff members who did the HPV tests were blinded to the results of the cervical Pap smears and biopsies, making a differential misclassification of viral load between cases and controls unlikely. These were likely to underestimate, rather than overestimate, our results. Our study was limited by the absence of an expert pathologist to confirm the biopsy pathology results although each cervical biopsy result was confirmed by two pathologists in each pathology department. An expert pathologic review may be needed to set up a good program in the future.

Another limitation of this study was that the Pap smears for HPV infection were sampled from the entire cervix whereas biopsy specimens were selected from several areas of cervix which would be considered for the most significant lesions by clinicians. The high positive rate (72%) for high-risk HPV infection in inflammatory biopsies was likely due to this limitation. In this study, we only measured specific HPV-16 infection and information was lacking on the frequency of other individual or multiple high-risk types of HPV infections. However, from the findings in Table 2, other high-risk HPV infection, in addition to HPV-16 infection, also played a pivotal role for the development of CIN risk in Taiwanese women.

In conclusion, high-risk HPV infection, irrespective of HPV-16 infection, is highly and positively associated with the development of CIN. The most high-risk HPV DNA load present in CIN2 women, but not in  $\geq$ CIN3 women, should be further investigated.

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