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人類疣瘤病毒與子宮頸癌變之巢疊病例對照研究(3/3)

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計畫主持人：陳建仁

計畫參與人員：陳慧祺, Dillner J, 孫建安, 游山林, Naucler P, Eklund C, 謝長堯

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中文摘要

子宮頸癌是台灣女性首位好發癌症，其年齡標準化發生率為每十萬人年 22.1 人，相較於已開發國家的平均發生率 11.2 人高出甚多。目前證據顯示主要危險因子為持續感染高危險型人類疣瘤病毒(human papillomavirus, HPV)。但是大多數的研究均屬於橫斷性病例對照研究，唯有透過長期追蹤研究才能釐清 HPV 與子宮頸鱗狀上皮內病變 (squamous intraepithelial lesion, SIL)的因果時序性。為探討人類疣瘤病毒感染與生殖道披衣菌感染對於誘發子宮頸癌的所扮演的角色及危險因子間的交互作用，本計畫採巢疊式病例對照研究設計，婦女研究世代源於 1991-1992 年在台灣七個鄉鎮市社區中邀請 30 至 64 歲婦女自願參加子宮頸癌篩檢計畫，收案時採集子宮頸抹片進行 SIL 之篩檢，並以陰道鏡輔助病理切片予以確診。相關危險因子係透過結構式問卷進行標準化訪視獲得。每名研究對象均以真空採血器及拋棄式針頭採取血液樣本，並以 Virapap 採取子宮頸細胞。續以全國癌症登記、子宮頸抹片檢查登記與死亡登記檔進行資料連鎖，追蹤該研究世代的新診斷子宮頸癌病例。追蹤至 2000 年共計 114 名病例，並依病例的受檢年齡、居住地區及受檢時間等條件進行配對，共獲得 519 名對照組個案。取出保存於低溫冷凍櫃中該研究個案受檢時所採集之血清/血漿進行 HPV6, 16, 18 等型抗體與披衣菌抗體檢測，並以子宮頸細胞進行 HPV DNA 檢驗。結果顯示 HPV16 型抗體陽性與子宮頸癌有著高度相關，其危險勝算比為 6.3 倍(95%信賴區間: 3.5-11.6)並達統計顯著差異。披衣菌感染與子宮頸癌的相關僅限於新發病例，其危險勝算比達 2.94 (95%信賴區間:1.17-7.42)。進一步分析顯示同時感染 HPV6 及 HPV16 與子宮頸癌的相關，呈現拮抗之交互作用，其相差危險性為 - 4.68 (95%信賴區間: -10.63 to 1.27)] ，同時感染 HPV16 及 HPV18 亦呈現類似的趨勢其相差危險性為 -3.27 (95%信賴區間: -7.71-1.18)。 本研究結果發現感染高危險性 HPV16 型與子宮頸癌之發生有高度相關，此外同時感染不同型別 HPV 或感染披衣菌者可能降低其致癌的風險。

ABSTRACT

Cervical cancer is the most common cancer among women in Taiwan with an age standardized incidence rate of 22.1 per 100,000 person-years compared to an average of 11.2 in developed countries. It is well established that the major cause of cervical cancer is persistent infection with oncogenic genital human papillomavirus (HPV). To investigate the possible role of interaction between human papillomavirus (HPV) types and past *Chlamydia trachomatis* exposure in cervical carcinogenesis in a population with low sexual risk-taking behavior, we performed a nested case-control study of invasive and *in situ* cervical cancer within a community-based cohort of 13,595 Taiwanese women assembled in 1991. During a 9 year follow-up period (1991-2000) incident cases were identified through linkage with nationwide cancer registry. Prevalent cases identified at the baseline cervical examination were also included in the study. Baseline serum or plasma from 114 cases and 519 controls matched for sex, age, area of residence, type of sample and date of enrolment were analyzed for antibodies against HPV types 6, 16 and 18 and against *C. trachomatis*. HPV-16 seropositivity was strongly associated with cervical cancer [OR= 6.33 (95% CI 3.45-11.62)]. *C. trachomatis* was overall not associated with cervical cancer, but was associated with cervical cancer in analyses restricted to incident cases of cancer [OR:2.94 (95% CI 1.17-7.42)] or to cases that had serum samples taken [OR:3.13 (95% CI 1.16-8.47)]. We found a tendency of antagonistic interaction between HPV-6 and -16 [Relative Excess Risk due to Interaction (RERI):- 4.68 (95% CI –10.63 to 1.27)] and between HPV-16 and -18 [RERI:-3.27 (95% CI -7.71-1.18)]. Our results suggest that different HPV types might interfere in cervical carcinogenesis, that *C. trachomatis* associates with cervical cancer in prospective studies and support the notion that HPV-16 seropositivity is strongly associated with cervical cancer risk in low sexual risk-taking populations.

INTRODUCTION

Cervical cancer is the most common cancer among women in Taiwan with an age standardized incidence rate of 22.1 per 100,000 person-years compared to an average of 11.2 in developed countries [1, 2]. It is well established that the major cause of cervical cancer is persistent infection with oncogenic genital human papillomavirus (HPV) [3] and that both cervical cancer and HPV infection are associated with high risk sexual behaviour [4, 5]. The HPV type distribution varies between different geographical regions but all over the world HPV 16 is the most common HPV type in cervical cancer[6, 7].

Since the majority of HPV infections are transient [8-10], viral genome detection is not a marker of past HPV infection[11]. However, HPV antibody levels have been shown to be stable over time even after more than a decade of follow-up [12, 13] and correlate with the life-time cumulative HPV exposure [13, 14]. Prospective seroepidemiological studies have found that presence of IgG antibodies against HPV 16 is associated with an increased risk of cervical cancer [13, 14], in particular in low sexual risk-taking populations[15, 16].

In spite of the fact that the exposure to oncogenic HPV increases with the number sexual partners, there is no excess risk of developing cervical cancer or cervical carcinoma in *situ* in patients with a history of *condylomata acuminata,* which is caused by sexually transmissible infections with the benign HPV types 6 and 11[17]. A possible explanation is that benign HPV types might interfere with the oncogenicity of high risk HPV types. Evidence of an antagonistic effect between benign and oncongenic HPV types has been found in seroepidemiological studies, where women seropositive for both HPV-6 and HPV-16 showed a significantly decreased risk of developing cervical cancer, compared to women seropositive for only HPV-16 [18-20].

Because only a small proportion of HPV-infected women actually develop cervical cancer [21], many epidemiological studies have been conducted to investigate additional risk factors that may be involved in the development of cervical cancer. Several studies have reported an association between past *Chlamydia trachomatis* infection and cervical cancer[22-24]. *C. trachomatis* is the most common sexually transmitted bacterial infection [25] and has been suggested to have a carcinogenic effect through modification of the ability to clear an HPV infection[26, 27].

Two previous case-control studies have assessed the association between HPV infection and cervical cancer in Taiwan, although only on a limited number of invasive cancer cases [28, 29]. Also, these studies have not investigated HPV serology and are therefore not informative regarding possible role of previous HPV infections. Since the peak prevalence of HPV infections occur when women initiate sexual relationships and cervical cancer develop 10-15 years after infection, most women that have been exposed to the virus and not developed disease will have cleared their infection when they are tested for the viral genome [30]. Although only about 50-65 % of HPV infected women seroconvert [31, 32], measurement of antibodies is still likely to reduce misclassification of past HPV exposure, when investigating additional co-factors to HPV in the development of cervical cancer.

To investigate the cervical cancer risk of past HPV and *C. trachomatis* exposures we therefore conducted a case-control study nested within a community-based cohort in Taiwan, where we measured the antibody response to HPV 6, 16 and 18 and *C. trachomatis* at baseline.

Material and methods

Study cohort

This Taiwan cohort was built based on a community-based cancer screening project (CBCSP), which provided two times of health examinations in the periods of 1991-1993 and 1993-1995. Seven townships were selected from 365 urban and rural administrative areas. There were 41,380 women aged from 30 to 64 registered in local household registration office y in 1990. They were invited by three consecutive mails.

There were a total of 13,595 women received at least once health examination from 1991 to 1995. Among them, 11,430 women had been examined by pap smear at least once. Table 1 showed the pap smears attendance rate by areas and age groups. The pap smear attendance rate ranged from 22.74% to 39.50%, with an overall attendance rate of 27.62%.

Data Linkage with National Profiles

Cervical cancer and carcinoma *in situ* (CIS) cases were identified through data linkage with national cancer registry, national death certification system and catastrophic illness registry with data available until December 31, 2000. Cases were further grouped as prevalent cases while she was registered as a cancer patient within one year after the date of her enrollment, or her biopsy was shown invasive cancer or carcinoma *in situ* at enrollment, or her pap smear already indicated as invasive cancer or carcinoma *in situ* at enrollment. Unsreened cases were defined since they didn't receive any pap smear at enrollment. Incident cases should have no cancer detected by pap smear at enrollment and be followed longer than one year to become a case.

Selection of Matched Case-control Sets

Six controls, who were found unaffected with the disease in these three data files, were randomly selected to match with each case on age, residence and within two months before/after the date of enrollment. Unaffected controls will be defined as "never being reported to have cervical neoplasia identified by Papanicolaou smear in National Cervical Neoplasia Registry, nor confirmed carcinoma in situ or invasive cancer of cervix uteri in National cancer Registry, nor death from cervical cancer in National Death Certification." The matching criteria will be "in the same five-year age group" for age and "within three months"

for date at recruitment. If more than four unaffected controls are available for a given newly diagnosed case, six controls with the age at recruitment nearest to the case will be selected.

We also refined the control group according their pap smear at enrollment. Eligible controls were defined, as her last pap smear was no cervical neoplsia at enrollment.

Specimens

Deep frozen biospecimens including serum and cervical cells collected in three consecutive examinations from 1991 to 1996 of these matched case-control sets will be retrieved and sent to the University of Lund in Sweden to test for seromarkers of chronic HPV infection and HPV DNA in cervical cells.

Chlamydia

Microimmunofluorescence: Chlamydia-specific lgG antibodies were detected using microimmunofluorescence as previously described [23, 37]. For *C. trachomatis* serovars D-K were used. *C. pneumoniae* serovar IOL 207 served as control antigen. All samples that were positive for titers ≥ 16 were further analysed in a titration series (16, 32, 64, 128 and 256). Titers of ≥ 64 were considered positive for both *C. trachomatis* and *C. pneumoniae*.

HPV serology testing

ELISA: Enzyme linked immunosorbent assay (ELISA) analysis was performed in Sweden to detect IgG antibodies against HPV types 6, 16 and 18 as previously described [33]. Virus-like particles (VLPs) that consist of self assembled L1 major capsid proteins generated in insect cells by recombinant baculovirus were coated onto ELISA plates [31]. Human antibodies against VLPs were detected using a two-step ELISA with monoclonal antibodies against human IgG and a goat anti-mouse IgG horseradish peroxidase conjugate. For each serum, the difference in optical density (OD) value obtained from plates coated with intact HPV VLPs and plates coated with control antigen (disrupted Bovine papillomavirus VLPs) was calculated. A seropositive reference serum, obtained from patients with cervical intraepithelial neoplasia, was used on each plate as a positive control and reference serum. All samples were screened at a 1/30 dilution where samples above the pre-assigned cut-off levels

for screening of 0.136 absorbance units for HPV-6 and 0.090 for HPV-16 [34], were selected for confirmatory analysis. Cut-off for HPV 18 was set as half the OD value for the reference serum. The confirmation and determination of antibody levels were performed using a serum titration series (1/10, 1/31.6, 1/100), where the antibody levels were calculated using the parallel line model (PLL) that expresses antibody levels as units relative to a reference serum tested on each plate [35]. The cut-off levels were, before the start of the study, set at 0.2445 units for HPV-6, to 0.3568 units for HPV-16 and 1.3678 units for HPV 18: The cut-off levels were for HPV16 and 18 set by treating cervical cancer as a receiver-operated characteristic using an independent population-based case-control study [36] and for HPV6 by treating HPV6 infection as the characteristic.

HPV DNA testing

Samples were analyzed by a general HPV primer $GP5^+/6^+$ -mediated PCR-EIA [Jacobs et al., 1997.]. Before analysis, the cells were thawed and centrifuged for 10min at 3,000g. The cell pellet was resuspended in 1 ml 10 mM Tris-HCL (pH 7.5) and frozen at –20℃. The samples were then thawed, and aliquots of 100 l were boiled for 10min; 10 l was used in a 50- l reaction mixture containing 200 M of each dNTP, 3.5 mM $MgCl_2$, 1U AmpliTaq DNA polymerase and reaction buffer (Perkin-Elmer, Foster City, CA), 0.5 M biotinylated $GP6^+$ primer, and 0.5 M $GP5^+$ primer. The quality of the sample DNA for amplification was analyzed in separate tubes, using a -globin PCR with biotinylated-BGPC03 and BGPC05 primers.

For the PCR, the laboratory of Malmo used a Hybaid OmniGene (Hybaid, Middlesex, UK) automated thermal cycler programmed for block temperature and Umea used a PTC-200 (MJ Research) in a "calculated control" mode. For PCR with GP^+ primers, a denaturation step at 94℃ for 4 min was followed by 40 cycles of amplification with segments at 94℃ for 1.5 min, 40℃ for 1.5 min, 72℃ for 2 min, and a final step at 72℃ for 4 min. The Stockholm laboratory used a GeneAmp 9700 (Perkin-Elmer) with a denaturation step at 94℃ for 4 min, followed by 40 cycles at 94℃ for 1 min, 38℃ for 1 min, 71℃ for 2min, and final step at 71℃ for 4 min. -Globin PCR was performed under the same conditions, but with 1.5 M $MgCl_2$ and an annealing temperature of 45℃.

As positive controls of -globin, an input of 1 ng and 10 ng human placental DNA (Sigma) was used. As positive controls of the HPV PCR, 10-foled dilutions of purified DNA

from SiHA cells were used, ranging from 10 ng to 100 pg of input DNA in a background of 100 ng human placental DNA (Sigma). As a negative control, 10 ml of water was added to the PCR and processed as the samples.

Typing of HPV

Aliquots (20 l) of PCR solutions from positive EIA samples were sent frozen for typing at a single laboratory (Malmo). HPV types were determined by the use of a non-radioactive reverse dot-blot hybridization. The membranes for use in the reverse dot-blot hybridization were prepared as follows. Recombinant HPV plasmids (100 ng DNA/dot), corresponding to the different HPV types tested for in the EIA, were denatured at high pH (0.8 M NaOH, 0.5 mM EDTA) for 20min and transferred by the use of a manifold (Schleicher & Schell, Dassel, Germany) to a prewetted (6X SSC) nylon membrane (Hybond N^+, Amersham, Buckinghanshire, UK). The membrane was neutralized with 200 l 20X SSPE, dried for 30 min at room temperature and baked at 120℃ for 20 min. Prehybridization of membranes was done for 1 hr at 46℃ in 5 ml solution containing 50% deionized formamide, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 1M NaCl, and 100 g/ml of herring sperm DNA in a hybridization oven (Hybaid); 5 l of PCR solution was added to 50 l of prehybridization solution, denaturated at 94℃ for 5 min and transferred to the prehybridization solution. After overnight hybridization, the membrane was rinsed once and then for 3X 15 min with 2X SSPE plus 0.1% SDS at 65℃ and incubated with 5 ml blocking solution at 65℃ for 1hr, consisting of 3% bovine serum albumin (BSA) in TBS-Tween (100 mM Tris-HCL, 150 mM NaCl, 0.05% v/v Tween 20, pH7.5), filtered through a sterile 0.45- M membrane (Acrodisc, Gelman Sciences, Ann Arbor, MI). The blocking solution was removed and the membrane incubated at room temperature for 10 min with 5 ml of streptavidin-alkaline phosphatase, (Gibco-BRL), diluted 1/3,300 in TBS-Tween, and filted through s sterile 0.22- M membrane (Millipore, Molsheim, France. The membrane was washed at room temperature for 2X10 min with TBS-Tween and finaly with 5 ml washing buffer (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5) for 1 hr. thereafter, the membrane was dried briefly on a filter paper to remove excess buffer, dipped in detection reagent (Lumin-Phos 530, Lumigen, Southfield, MI) and placed in a transparent folder, and incubated for 1.5 hr at room temperature. The membrane was exposed to X-ray film (Kodak X-AR, Kodak, Rochester, NY) in a cassette with intensifying screens (Du Pont, Cronex lightning plus, NEN, Boston, MA) for 10 min. An HPV type was considered identified when a

clear-cut spot of darkening of the film could be distinguished from the background.

Data analysis

SAS statistical software was used for data linkage and analysis. Odds ratios (ORs) and corresponding 95 % confidence intervals (CIs) were estimated in univariate and multivariate conditional logistic regression models. Selected variables for inclusion in the multivariate model were variables that were significantly associated with cervical cancer when all variables where included in the adjusted logistic regression model. In addition, the matching variables and variables that have previously been described to be associated with cervical cancer were included. The analysis of interaction between different HPV infections was estimated using unconditional logistic regression, by calculating ORs with 95 % CIs for cervical carcinoma among subjects exposed to one or two HPV infections using the jointly un-exposed subjects as reference [19]. The observed risk was compared to the expected risk using an additive model where the relative excess risk due to interaction (RERI) is the relative risk (RR) when exposed to both causes A and B, RR(AB), minus the RR when exposed to only the first cause (A*) minus the RR when exposed to only the second cause (*B) plus 1 [RERI= RR(AB)-RR(A*)-RR(*B)+1] [38]). In the absence of interaction RERI equals zero and there is evidence of interaction if the 95% confidence interval excludes zero [39].

RESULTS

Socio-demographic characteristics of cases and controls are presented in Table 1. The mean age at baseline was 48.3 years for cases and 47.8 years for controls. Women with invasive cervical cancer tended to be older than women with carcinoma in situ (mean age at baseline was 50.4 and 45.9 respectively). Only ten study subjects reported a history of smoking. Thirty-seven percent of the subjects were illiterate, 43 % had only been to primary school and less than one percent had been to university. Out of 633 study subjects there were 603 who reported having had only one lifetime sexual partner. The median age at first intercourse was 21 for cases and 22 for controls. Thirty-three percent of cases and 37% of controls reported having had a Pap smear taken earlier in life before enrolment. Former use of oral contraceptives was reported by 33 % of cases and 27 % of controls (Table 1).

Antibodies against HPV 16 were strongly associated with cervical cancer [Adjusted OR= 6.33 (95% CI 3.45-11.62)] (Table 2). HPV 16 antibodies were more common among carcinoma *in situ* cases (33.3 %) than among invasive cancer cases (25.0%) (Table 2). However, the HPV 16 associated risk did not differ between invasive cervical cancer and carcinoma *in situ* [Adjusted OR: 6.35 (95% CI 2.62-15.41) and 6.25 (95% CI 2.74-14.28), respectively]. HPV-6 was the type most frequently found in both cases (37.7 %) and controls (31.0%), but there was no elevated cervical cancer risk for women with past infection with HPV-6 [Adjusted OR: 1.07 (95% CI 0.65-1.77)]. Neither was there an elevated risk among HPV-18 positive subjects [Adjusted OR: 0.60 (95% CI 0.30-1.23)]. There was no significant difference in HPV 6 and HPV 18 prevalences between invasive cancer cases (41.7 % and 20.0 % percent respectively) and carcinoma *in situ* cases (33.3 % and 14.8 % respectively) and neither infection was associated with invasive cancer or carcinoma *in situ*. Except for past HPV-16 infection, history of smoking was the only variable that was significantly associated with cervical cancer in the multivariate model [Adjusted OR: 4.92 (95% CI 1.06-22.84)].

When prevalent, incident and unscreened cases were analyzed separately, past HPV 16 infection was significantly associated with cervical cancer in all three subgroups (Table 3). HPV 6 was significantly associated with cervical cancer among incident cases [Adjusted OR: 2.68 (95% CI 1.05-6.86)], but not among prevalent cases [Adjusted OR: 0.63 (95% CI 0.33-1.18)]. HPV 18 was neither associated with prevalent or incident cases. The adjusted OR for unscreened cases could not be calculated because of the low number of cases.

Interaction between HPV 6, 16 and 18 was investigated using the jointly un-exposed subjects as reference (Table 4). There appeared to be a decreased risk of cervical cancer among women exposed to both HPV 6 and 16, compared to women exposed to HPV 16 only. The relative excess risk due to interaction (RERI) was -4.68 (95% CI -10.63 to 1.27). There also appeared to be a reduced risk for women exposed to both HPV 16 and 18 compared to women exposed to HPV 16 only [RERI: -3.27 (95% CI -7.71 to 1.18)]. There was no tendency for interaction between HPV 6 and 18 [RERI: 0.28 (95% CI -1.13 to 1.70)]. There were little difference in the point estimates when adjusted for age, area, history of smoking, C. *trachomatis* and other HPV type.

Antibodies against *C. trachomatis* were not significantly associated with cervical cancer [Adjusted OR: 1.54 (95% CI (0.93-2.55)] when all cases where included in the analysis (Table 5). Neither was *C. trachomatis* associated with invasive cancer [Adjusted OR: 1.64 (0.83-3.23)] or carcinoma in situ [Adjusted OR: 1.41 (0.65-3.04)]. A significant association with cervical carcinoma was observed among incident cases [Adjusted OR: 2.94 (95% CI 1.17-7.42)] but not among prevalent cases [Adjusted OR: 1.14 (95% CI 0.62-2.10)] (Table 5). When analyses were restricted to subjects with serum or plasma samples, an association with cervical cancer was evident among women who had serum samples taken [Adjusted OR: 3.13 (95% CI (1.16-8.47)] compared to women with plasma samples [Adjusted OR: 1.13 (95% CI 0.62-2.07)] (not shown in table; adjusted for age (50), area, ever use of oral contraceptives, history of smoking and seropositivity for HPV 6, 16 and 18). There was a significant difference in the prevalence of past *C. trachomatis* infection among controls (χ^2-test p: 0.025) but not among cases (χ^2-test p: 0.383), when subjects with serum and plasma where compared. *C. pneumoniae* was not associated with cervical cancer [Crude OR: 0.90 (95% CI 0.65-2.15)] (not shown in table). There was no difference in the prevalence of *C. pneumoniae* among cases (χ^2-test p: 0.417) or controls (χ^2-test p: 0.643) when serum and plasma were compared. To test for cross-reactivity between *C. trachomatis* and *C. pneumoniae*, the proportion of *C. pneumoniae* positive samples was compared in *C. trachomatis* positive and negative samples. A significantly increased proportion was observed in plasma samples (χ^2-test p: 0.002), but not among serum samples (χ^2-test p: 0.688).

DISCUSSION

In accordance with previous seroepidemiolgical studies, we found that HPV 16 seropositivity was significantly associated with cervical cancer [16, 33]. The strongly elevated risk in our study [Adjusted OR= 6.33 (95% CI 3.45-11.62)] is well in line with the low exposure level in the background population: only 8% of controls were seropositive for HPV 16. This is lower than has been observed in other studies [19, 40, 41]. We have previously reported that the HPV 16 associated risk of developing cervical cancer is higher in populations with a low background seroprevalence of HPV 16 and *C. trachomatis* than in populations with a high background prevalence[16]. We have suggested that when most of the population is HPV exposed, mere HPV exposure is no longer the major environmental risk factor and cofactors will be comparatively more important in determining which women actually develop disease[16]. In such highly exposed populations, the proportion of HPV-negatives that are false negative is increased, thereby diluting risk estimates. The low exposure level of HPV 16 infection among controls in this study is likely to reflect that HPV 16 is a rare sexually transmitted infection in Taiwan. Liaw et al. who analyzed 261 cytologically normal women from the same cohort, found that only 0.8% were HPV DNA positive for high risk HPV types 16, 18, 31 or 45 [28].

In contrast to the low HPV 16 prevalence, we found that the HPV 6 seroprevalence was 31% among controls which is even higher than among controls in many other case-control studies [19, 41]. The frequency of controls being infected by HPV 18 was essentially similar to other case-control studies [19, 40]. The high prevalence of HPV 6 and 18 among controls, even though only 2.7 % of the women reported having had more than one lifetime sexual partner, might be due to the sexual behaviour of the husbands. A previous population based case-control study from Taiwan reported that 71.9% and 58.3% of husbands to women with CIN and healthy controls respectively, had visited a prostitute during the last year [42]. Another plausible explanation is the presence of alternative transmission routes in this population. Liaw et al. found that 39% of HPV DNA positives cases with high grade squamous intraepithelial lesions to be positive with HPV 52 and/or 58, which is a higher prevalence than reported from other geographical areas [28, 43]. We were unfortunately not able to test for antibodies to HPV 52 and 58.

Incident cases tended to be invasive rather than *in situ* cancers (66% invasive cancers), whereas the opposite was true for prevalent cases (44% invasive cancers). This is most likely

attributable to the fact that prevalent cases were recognised at the baseline examination that included a pap smear, whereas the incident cases were recognized through linkage with the national cancer registry, the national death certification system and catastrophic illness registry where both symptomatic and screen-detected disease would have been registered.

We performed analyses on incident cases and prevalent cases separately, because prospective data is more informative on possible etiologic role of associations. HPV 16 was the only infection associated with cervical carcinoma among prevalent cases, whereas among incident cases, both *C. trachomatis*, HPV 6 and HPV 16 were associated with disease. An explanation is not immediately obvious, but possibly the more reliable prospective analyses had an increased ability to detect a cofactor role of sexual high risk taking behaviour.

We did not find any difference in the HPV types associated with invasive cancer compared to carcinoma *in situ*, HPV 16 being the only type associated with disease. A history of smoking was estimated to have a high risk for cervical cancer, but because of the low overall prevalence of smoking the confidence interval was wide.

Despite the high point estimates of an antagonistic effect in cervical carcinogenesis between HPV 6 and 16 and between HPV 16 and 18, the effect was not statistically significant. However, the tendencies found are in concordance with previous studies that have demonstrated not only an antagonistic effect between HPV 6 and 16, but also reported a tendency of interaction between HPV 16 and 18 [18-20]. The point estimates of interaction in an adjusted analysis including history of smoking, *C. trachomatis* infection and other HPV infection were consistent with the estimates obtained in the univariate analysis.

Future studies that investigate interaction between HPV types, would require larger case groups, since statistical power when studying interaction requires much larger studies than when studying the primary effect of a risk factor. Our study only included serological analyses of three HPV types. Taking into account the difference in geographical distribution of HPV types and that 15 types are considered as high-risk types [44] it would also be desirable to conduct studies where the antibody response to more HPV types are investigated to obtain a more complete picture of interactions between HPV types. Probably, the most informative study design to elucidate if one HPV infection protects against persistence of another HPV infection would be to test a cohort of HPV DNA positive subjects at base-line for HPV seropositivities to other types and assess if these seropositivities predict clearance of HPV

infections.

Inter-assay variability can be a problem in serological studies, since ELISA-methods are sensitive to the reaction conditions. When optical density values are used as cut-off level, strictly standardized reaction conditions are required, which may be difficult to achieve. We have previously demonstrated that measurement of antibody levels in parallel line model (PLL) units relative to an internal standard, significantly reduces the inter-assay variability and allows comparison of results from different studies[35].

C. trachomatis was not significantly associated with cervical cancer in our study. This was surprising, since many other studies have found an increased risk of cervical cancer for women with antibodies against *C. trachomatis* both in analyses that have adjusted for HPV infection and in stratified analyses where only HPV DNA positive cases and controls have been included [23, 24]. However, whereas there was no increased risk among prevalent cases, we did find an increased risk among incident cases, which is not explained by the higher proportion of invasive cancer among incident cases [Crude OR: 2.97 (95% CI 1.36-6.51) for incident invasive cancers and Crude OR: 3.15 (95% CI 0.83-11.97 for incident carcinoma *in situ*]. Whereas 17 % of the prevalent cases and corresponding matched controls had serum samples taken, as many as 62 % of the incident cases and corresponding matched controls had serum samples taken. Existence of cross-reactivity between *C. trachomatis* and *C. pneumoniae* in plasma samples was suggested by significant clustering of seropositivties for these 2 agents, which was not detected in serum samples. Similar analysis comparing HPV prevalences in serum and plasma did not find any clustering, neither among cases or controls. Microimmunofluorescence is a distinct method (the entire C. *trachomatis* organism is used) from ELISA, and a conceivable explanation for our findings is that there might be a non-specific reactivity with *C. trachomatis* antigen when plasma is analysed which decreases the specificity of the test. An alternative explanation is the known fact that *C. trachomatis* antibodies decline over time, which would make earlier, prediagnostic measurements more accurate. Finally, it is also possible that *C. trachomatis* only has an effect many years before cancer and that non-causative *C. trachomatis* exposures occurring close to diagnosis (too short lag time for an effect) will dilute associations in studies of prevalent cases.

Despite that more than 60% of the women in the study reported that they never had had a Pap smear taken, we did not find any protective effect of having had a Pap smear taken,

neither in crude or adjusted models. We did not obtain information on the numbers or regularity of Pap smears taken and thus it is possible that many had only had a Pap smear taken once. Because the sensitivity of Pap smears for detecting cervical intraepithelial neoplasia is limited and varies in different studies (from 30-87%), it is possible that the protective effect of a few smears may have been to small to detect [45].

In conclusion, women with past infections of both HPV 6 and HPV 16 or HPV 16 and 18 seem to have a decreased risk of developing cervical cancer compared to women with past infection of only HPV 16, but further and larger studies will be necessary to fully understand the interplay between HPV types in cervical carcinogenesis. We confirm that *C. trachomatis* associates with cervical cancer in prospective studies. Our lack of association in the ordinary case-control study of prevalent cases has highlighted several possible methodological problems that needs to be considered in further studies of *C. trachomatis* and cancer. Finally, the fact that HPV 16 seropositivity was so strongly associated with cervical cancer supports the view that in this low sexual risk population, HPV16 exposure *per se* is a major limiting factor determining cervical cancer incidence.

Table 1 Distribution of socio-demographic characteristics among cases and matched controls nested within a Taiwanese cohort of 13 595 women.

Socio-demographic Characteristic	**Controls**	**All cases**	**Carcinoma in situ**	**Invasive Cancer**
Total	519	114	54	60
Age (years)				
30-39	115 (22.2)	27 (23.7)	15 (27.8)	12 (20.0)
40-49	171 (32.9)	32 (28.0)	20 (37.0)	12 (20.0)
50-64	233 (44.9)	55 (48.2)	19 (35.2)	36 (60.0)
Education				
Illiterate	186 (35.8)	48 (42.1)	19 (35.2)	29 (48.3)
Primary, Middle or High School	314 (60.5)	63 (55.3)	34 (63.0)	29 (48.3)
Higher Education	18 (3.5)	2 (1.8)	1 (1.9)	1 (1.7)
Information missing	1 (0.2)	1 (0.9)	0	1 (1.7)
History of smoking				
Yes	5 (1.0)	5 (4.4)	2 (3.7)	3 (5.0)
No	513 (98.8)	108 (94.7)	52 (96.3)	51 (93.3)
Information missing	1 (0.2)	1 (0.9)	0	1 (1.7)
Age at sexual debute (years)				
≤18	37 (7.1)	10 (8.8)	8 (14.8)	2 (3.3)
19-26	433 (83.4)	93 (81.6)	41 (75.9)	52 (86.7)
≥27	44 (8.5)	3 (2.6)	2 (3.7)	1 (1.7)
Information missing	5 (1.0)	8 (7.0)	3 (5.6)	5 (8.3)
Number of lifetime sexual partners				
1	501 (96.5)	102 (89.5)	49 (90.7)	53 (88.3)

≥2	14 (2.7)	4 (3.5)	2 (3.7)	2 (3.3)
Information missing	4 (0.8)	8 (7.0)	3 (5.6)	5 (8.3)
Self-reported genital warts				
Yes	3 (0.6)	1 (0.9)	1 (1.9)	0
No	485 (93.4)	95 (83.3)	47 (87.0)	48 (80.0)
Information missing	31 (6.0)	18 (15.8)	6 (11.1)	12 (20.0)
Oral contraceptives ever				
Yes	140 (27.0)	38 (33.3)	22 (40.7)	16 (26.7)
No	375 (72.3)	68 (59.6)	29 (53.7)	39 (65.0)
Information missing	4 (0.8)	8 (7.0)	3 (5.6)	5 (8.3)
Pap smear ever taken before enrolment				
Yes	191 (36.8)	37 (32.5)	19 (35.2)	18 (30.0)
No	322 (62.0)	69 (60.5)	32 (59.3)	37 (61.7)
Information missing	6 (1.2)	8 (7.0)	3 (5.6)	5 (8.3)

Table 2 Risk of cervical carcinoma in Taiwanese women with antibodies against HPV 6, 16 and 18

	Risk in all women with cervical carcinoma (n=114)				**Risk in women with invasive cancer** (n=60)	**Risk in women with carcinoma *in situ*** (n=54)
HPV serology	**Case** (n=114)	**Controls** (n=519)	**Crude OR** (95% CI)	**Adjusted OR*** (95% CI)	**Adjusted OR**** (95% CI)	**Adjusted OR**** (95% CI)
HPV 6	43 (37.7%)	161 (31.0%)	1.25 (0.80-1.95)	1.07 (0.65-1.77)	1.00 (0.48-2.10)	0.93 (0.46-1.88)
HPV 16	33 (28.9%)	42 (8.1%)	5.43 (3.09-9.53)	6.33 (3.45-11.62)	6.35 (2.62-15.41)	6.25 (2.74-14.28)
HPV 18	20 (17.5%)	77 (14.8%)	1.26 (0.71-2.26)	0.60 (0.30-1.23)	0.74 (0.29-1.91)	0.43 (0.14-1.34)

* Adjusted for age (five year intervals), area, ever use of oral contraceptives, history of smoking, seropositivity for C. trachomatis and seropositivity for other HPV types.

** Adjusted for age (50), area, history of smoking, ever use of oral contraceptives, seropositivity for C. trachomatis and seropositivity for other HPV types.

Table 3 HPV 6, 16 and 18 associated risk in Prevalent, Incident and Unscreened cervical cancer cases

HPV serology	**Prevalent Cases**	**Incident Cases**	**Unscreened Cases**
	(n=72)	(n=35)	(n=7)
	Adjusted OR* (95% CI)	Adjusted OR* (95% CI)	Crude OR (95% CI)
HPV 6	0.63 (0.33-1.18)	2.68 (1.05-6.86)	0.82 (0.050-13.24)
HPV 16	8.12 (3.91-16.83)	3.78 (1.13-12.60)	not analysable
HPV 18	0.58 (0.25-1.38)	0.41 (0.082-2.06)	not analysable

* Adjusted for age (50), area, ever use of oral contraceptives, seropositivity for C. trachomatis and seropositivity for other HPV types.

Table 4 Joint-effects of HPV-6, HPV-16 and HPV-18 in women with cervical carcinoma.

		Cases	**Controls**	**OR** (95 % CI)	**Adjusted OR*** (95 % CI)
HPV-6	**HPV-16**				
No	No	52	342	1	1
Yes	No	29	135	1.41 (0.86-2.32)	1.22 (0.69-2.14)
No	Yes	19	16	7.81 (3.78-16.15)	10.52 (4.72-23.45)
Yes	Yes	14	26	3.54 (1.74-7.22)	5.50 (2.35-12.89)
HPV-6	**HPV-18**				
No	No	63	319	1	1
Yes	No	31	123	1.28 (0.79-2.06)	1.00 (0.59-1.73)
No	Yes	8	39	1.04 (0.46-2.33)	0.51 (0.19-1.40)
Yes	Yes	12	38	1.60 (0.79-3.23)	0.43 (0.17-1.08)
HPV-16	**HPV-18**				
No	No	71	420	1	1
Yes	No	23	22	6.18 (3.28-11.69)	7.55 (3.84-14.85)
No	Yes	10	57	1.04 (0.51-2.13)	0.57 (0.23-1.41)
Yes	Yes	10	20	2.96 (1.33-6.58)	3.19 (1.34-7.60)

RERI (HPV6 and HPV16): 3.54 -7.81-1.41+1= - 4.68 (95% CI –10.63 to 1.27)

RERI (HPV6 and HPV18): 1.60-1.28-1.04+1= 0.28 (95% CI -1.13 to 1.70)

RERI (HPV16 and HPV18): 2.96-6.18-1.04+1= -3.27 (95% CI -7.71 to 1.18)

* Adjusted for age, area, ever use of oral contraceptives, history of smoking, seropositivity for C. *trachomatis* and seropositivity for other HPV type.

Table 5 Cervical cancer risk in women with past infection of *C. trachomatis*

C. trachomatis	**Cases** Positive	**Controls** Positive	**Crude OR** (95% CI)	**Adjusted OR** (95% CI)
All cases	41 (36.0%)	149 (28.7%)	1.39 (0.88-2.20)	1.54 (0.93-2.55) *
Prevalent cases (n=72)	23 (31.9%)	115 (31.4%)	1.06 (0.60-1.88)	1.14 (0.62-2.10) **
Incident cases (n=35)	15 (42.9%)	29 (20.1%)	2.85 (1.27-6.44)	2.94(1.17-7.42) **
Unscreened cases (n=7)	3 (42.9%)	5 (55.6%)	0.37 (0.030-4.63)	Not applicable
Carcinoma *in situ* (n=54)	17 (31,5%)	68 (26,2%)	1,24 (0,63-2,44)	1,41 (0,65-3,04) **
Invasive Cancer (n=60)	24 (40,0%)	81 (31,3%)	1,49 (0,80-2,78)	1,64 (0,83-3,23) **

* Adjusted for age (five year intervals), area, ever use of oral contraceptives, history of smoking, seropositivity for C. trachomatis and seropositivity for HPV 6, 16 and 18.

** Adjusted for age (50), area, ever use of oral contraceptives and seropositivity for HPV 6, 16 and 18.

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