行政院國家科學委員會專題研究計畫　期中進度報告

人類疣瘤病毒與子宮頸癌變之巢疊病例對照研究(2/3)

計畫類別：個別型計畫
計畫編號：NSC92-2320-B-002-136-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學公共衛生學院流行病學研究所

計畫主持人：陳建仁

報告類型：精簡報告
報告附件：國際合作計畫研究心得報告
處理方式：本計畫可公開查詢

中　華　民　國 93 年 5 月 31 日

行政院國家科學委員會

一般型研究計畫

人類疣瘤病毒與子宮頸癌變之巢疊病例對照研究

九十二年度期中報告

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計畫編號：NSC 91-2320-B-002 –076 –

執行期限：92/08/01 ~ 93/07/31

Cervical cancer is the leading cancer among women in Taiwan (24% of all incident cancers in women) with an annual age-adjusted incident rate of 51.0 per 100,000 (Cancer Registry Annual Report, Republic of China, 1999), which is about five times higher than that in the United States (8.7 per 100,000) (Miller et al., 1993). Reasons for the high risk in Taiwan are unclear, since the reported prevalence of known epidemiologic risk factors for cervical cancer, in particular multiple sexual partners, is quite low compared with Western women. However, more than 90% of the women in Taiwan had never had a Papanicolaou (Pap) smear before 1990 (Health Annual Report in Taiwan, 1993), which might be related to the high risk. As reported in Western countries, a clinical report from China and Taiwan has shown that HPV could be detected in over 70% of cervical cancer patients, suggesting that HPV may be an important risk factor for cervical cancer in this monogamous female population.

There were only few long-term follow-up studies on HPV and cervical neoplasia in other countries, and no long-term follow-up study has ever been carried out in Taiwan. This study, nested case-control study based on a large-scale community-based cervical neoplasia screening project, will be undertaken to evaluate the role of HPV infection and other risk factors in the development of cervical neoplasia in Taiwan.

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. For *C. trachomatis*, titers against serovars (serotypes) D-K were determined. *C. pneumoniae* serovar IOL 207 served as control antigen. Titers of ≥32 were considered positive.

HPV serology testing

ELISA: Enzyme linked immunosorbent assay (ELISA) methodology was used to detect antibodies against HPV types 6, 16 and 18 as previously described. Briefly, virus-like particles (VLPs), self assembled L1 major capsid proteins generated in insect cells by recombinant baculovirus were coated onto ELISA plates: HPV-6 VLPs at 0,5 μg/ml, HPV-16 VLPs at 0,25 μg/ml, HPV-18 VLPs at 0,5 μg/ml and bovine papillomavirus (BPV) VLPs at 0,5 μg/ml. Human antibodies against VLPs were detected using 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 obtained with plates coated with intact HPV VLPs and plates coated with control antigens (disrupted BPV VLPs) was calculated. To select which samples that should be tested in a serum titration series (1/10, 1/31.6, 1/100), ELISA was performed on all samples in 1/30 dilution. All samples with optical density value above the pre-assigned cut-off level of 0,136 for HPV-6, 0,306 for HPV-18 and 0,090 for HPV-16, were selected. The parallel line model (PLL) was used to determine a PLL unit for each titrated sample. A seropositive reference serum was used on each plate. Cut-off levels for seropositivity were pre-assigned and set to 0,2445 units for HPV-6. For HPV-16 and HPV-18 both disease-specific and infection-specific cut-off levels were used. The cut-off levels were 0,3568 and 0,1650 units for HPV16 and 1,3678 and 0,5384 units for HPV-18 respectively. The infection specific cut-off level is based on a virgin pool were cut-off is set to the mean value of the pool plus three standard deviations. The disease specific cut-off level is based on treating cervical cancer as a receiver-operated 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°C. The samples were then thawed, and aliquots of 100 µl were boiled for 10min; 10 µl was used in a 50-µl reaction mixture containing 200 µM of each dNTP, 3.5 mM $MgCl_2$, 1U AmpliTaq DNA polymerase and reaction buffer (Perkin-Elmer, Foster City, CA), 0.5 µM biotinylated $GP6^+$ primer, and 0.5 µM $GP5^+$ primer. The quality of the sample DNA for amplification was analyzed in separate tubes, using a β-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°C for 4 min was followed by 40 cycles of amplification with segments at 94°C for 1.5 min, 40°C for 1.5 min, 72°C for 2 min, and a final step at 72°C for 4 min. The Stockholm laboratory used a GeneAmp 9700 (Perkin-Elmer) with a denaturation step at 94°C for 4 min, followed by 40 cycles at 94°C for 1 min, 38°C for 1 min, 71°C for 2min, and final step at 71 °C for 4 min. β-Globin PCR was performed under the same conditions, but with 1.5 µM $MgCl_2$ and an annealing temperature of 45°C.

As positive controls of β-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 (20μl) 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 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 μl 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 μg/ml of herring sperm DNA in a hybridization oven (Hybaid); 5 μl of PCR solution was added to 50 μl 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-μM 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-μM 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. The relative risks (with 95 % confidence intervals) of HPV infection and cervical neoplasia were calculated by logistic regression model. $\mathbf{RR_A}$ indicates that relative risks were estimated after age and area adjusted in unconditional logistic regression model. $\mathbf{RR_M}$ represents relative risks, which were estimated in matched analysis by Cox's proportion hazard model. Other risk factors on cervical neoplasia will be further analyzed.

Results

After linkage with national cancer registry, national death certification system and catastrophic illness registry, there 122 cases were registered as cervical cancer. In the original sampling sets, we proposed to have 700 control were matched for cases. 740 of them had been sent to Sweden for testing of HPV serology. The other specimen need to been picked out from the serum bank. After exclusion, there were 114 cases and 519 controls were performed for further analyses.

As heretofore, the serologic testing of HPV type 6 and type 16 were completed, and result of type 18 needs to be confirmed for quality control. This preliminary result of HPV serology has been reported in last year. HPV type 16 infections increased the risk of cervical cancer developing. However HPV serology positivity presents a cumulative phenomenon of viral infection and of HPV DNA presents the active status of viral infection, we tried to test HPV DNA and typing in the coming year.

We have HPV DNA and typing results of 132 samples, including 25 cases and 107 matched controls. There were 18 cases and 19 controls shown positive in the detection of HPV DNA. HPV DNA positive rate were 72% and 17.8% in cases and controls respectively. The positives were further typing and the result was listed in table 1.

Table 1: The distribution of HPV DNA and typing in positives.

	Case (n=18)	Control (n=19)	Total
Single infection			
16	6	2	8
18	2	0	2
31	0	1	1
33	2	3	5
35	0	2	2
45	1	2	3
51	0	1	1
52	2	1	3
56	0	3	3
58	3	1	4
66	1	0	1
Multiple infection			
16,56	0	1	1
18,52	1	0	1
31,59	0	1	1
39,59	1	0	1

Although the amount of sample was small, the result still showed the strong association between HPV DNA and cervical neoplasia, especially to HPV type 16. We will keep working on the experiments in the coming year.