

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(1/3)

計畫類別：個別型計畫

計畫編號：NSC91-2320-B-002-076-

執行期間：91年08月01日至92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

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中華民國 92 年 6 月 2 日

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 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. Unscreened 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 neoplasia 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.

HPV DNA testing

The cervical cell samples will be tested for presence of HPV DNA of types 6,11,16,18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 66, 68 and a cocktail of clinical types for which a type number has not been assigned at the time of testing. Testing will be done at Prof. Joakim Dillner's laboratory in the University of Lund, Sweden using the L1 consensus primer PCR-based method. The amplification reactions included modified consensus primer pair MY09*/MY11. Amplification products were hybridized with a generic HPV probe mixture to determine the positivity, and with specific oligonucleotide probes to identify individual types. In the PCR, a set of internal control primers amplifying the β -globin gene will be included to monitor sample sufficiency and the absence of PCR

inhibition. In addition, positive (HeLa cervical cancer cell line) and negative controls (K562 human cell line) will be incorporated into the PCR process. The assay reproducibility will be measured by retesting 10% of randomly selected samples.

In the analysis, the various types of HPV will be combined into three groups according to their prevalence in cervical cancer based on a large-scale international study. The high-risk types (16,18, 31 or 45) have been detected in 77% of cervical cancer samples worldwide. The medium-risk types (26, 33, 35, 39, 51, 52, 55, 56, 58, 59 or 68) have less commonly be associated with cervical cancer. The low-risk types (6, 11, 40, 42, 53, 54 or 57) have not generally been found in cervical cancer. The low-risk group will also include the clinical types, unknown types (PCR product hybridized with the consensus probe but none of the type-specific probes) and undetermined types (specimens whose HPV DNA level was too low to permit typing). Subjects with infection by multiple types of HPV will be assigned hierarchically to the highest risk group applicable.

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.

Chlamydia

Microimmunofluorescence: Chlamydia specific IgG 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.

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. **RR_A** indicates that relative risks were estimated after age and area adjusted in unconditional logistic regression model. **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.

Preliminary 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. We will have HPV DNA testing in this coming year. Testing of Chlamydia is ongoing smoothly, and we will have the result in near future. Serologic HPV type 6 was found 48.3% in case and serology positivity

The HPV serology-type-specific positive rates in the control group were 41.0%, 10.4% and 46.2% for type 6, 16, and 18 respectively. In the case group, 48.3%, 33.3% and 53.5% of women were found to be positive of HPV serologic type 6, 16 and 18. The relative risks of HPV type 6 was 1.3 (95%CI: 0.9-2.0), which is non-significantly higher than the control group and seems to be constant after age and area adjustment using unmatched or matched analyses. For type 16, the case group have statistically significant four-fold risk to be positive relative to the control, both **RR_A** and **RR_M** are also significantly higher as 4.3 (95%CI: 2.7-7.1) and 4.9 (95%CI: 2.8-8.4). The RRs of HPV type 18 were similar with type 6, which were listed in Table 2.

Since the serum specimen were collected at enrollment and the serologic positivity of HPV would indicate a previous infection experience of a woman. Table 3 showed that women with previous infection of HPV type 16 have 4.6 times risk (95%CI: 2.6-8.4) for whom were diagnosed as prevalent cases at enrollment, including invasive cervical cancer and carcinoma *in situ*. It also showed a statistically significant risk (**RR_A**: 3.9, 95%CI: 1.7-8.8) for women with previous infection of HPV type 16 to incidental cervical cancer developing during subsequent follow-up. HPV type 6 infections didn't play important role in prevalent cervical cancer patients, but it pointed out three-fold risk in development of cervical cancer

This preliminary result strongly supports that HPV infection increased the risk of cervical cancer developing, especially HPV type 16 is. However HPV serology positivity presents a cumulative phenomenon of viral infection and of HPV DNA presents the active status of viral infection, we need to incorporate both of them to elucidate the association of HPV infection on the risk of cervical neoplasia.

Table 1: Attendance rate of CBCSP Taiwan cohort

	Invited women (N=41,380)	Attendance for CBCSP (N=13,595)	Attendance for pap smear screening (N=11,430)	Pap smear attendance rate % (27.62)
Area				
SJ	2817	817	779	27.65
GD	11959	3194	3003	25.11
PZ	8031	1873	1826	22.74
KS	5307	2819	2096	39.50
MK	9590	3045	2864	29.86
HS & BS*	3676	1847	862	23.45
Age				
30-34	7412	1830	1652	22.29
35-39	7538	2267	2043	27.10
40-44	6195	1953	1712	27.64
45-49	4875	1779	1493	30.63
50-54	5691	2206	1774	31.17
55-59	5591	2072	1631	29.17
60-64	4078	1488	1125	27.59

*: Two neighboring townships (HS & BS) were combined for analysis

Table 2: Risks of HPV type 6, 16 and 18 serology positivity and cervical cancer in case-control study

HPV Serology	Case (n=114)	Control (n=519)	RR	RR _A	RR _M
Type 6	55(48.3)	213(41.0)	1.3(0.9-2.0)	1.3(0.8-1.9)	1.3(0.8-1.9)
Type 16	38(33.3)	54(10.4)	4.3(2.7-7.0)	4.3(2.6-7.1)	4.9(2.8-8.4)
Type 18	61(53.5)	240(46.2)	1.3(0.9-2.0)	1.3(0.9-2.0)	1.4(0.9-2.1)

RR_A: relative risks were estimated after age and area adjusted in unconditional logistic regression model

RR_M: relative risks were estimated in matched analysis

Table 3: Risks of HPV type 6, 16 and 18 serology positivity and cervical cancer in case-control study

HPV	Prevalent cases (n=72)	Incident cases (n=35)	Unscreened cases (n=7)
Serology	RR_A	RR_A	RR_A
Type 6	0.9(0.5-1.5)	3.0(1.4-6.4)	1.9(0.4- 8.6)
Type 16	4.6(2.6-8.4)	3.9(1.7-8.8)	3.4(0.7-18.2)
Type 18	1.3(0.8-2.2)	1.2(0.6-2.4)	2.9(0.6-15.1)

RR_A: relative risks were estimated after age and area adjusted in unconditional logistic regression model