The clinical significance of allelic alteration of TSG loci in cervical intraepithelial neoplasia

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The involvement of the HPV in the development of cervical cancer has been firmly established. Specific human papillomavirus (HPV) types appear to be necessary etiological factors for most cervical cancers. High-risk HPVs such as HPV16 and HPV18 are causative agents for high-grade intraepithelial neoplasia (CIN3) and cervical cancer. One mechanism by which the virus contributes to disease progression is by causing genetic instability of the host genome [Hashida et al 1992; White et al 1994]. This is explained in part by the interaction of the viral oncoproteins E6 and E7 with key cell regulatory proteins such as p53 and pRb thereby deregulating the cell cycle, cell differentiation, DNA repair, and apoptosis [Scheffner et al 1990; Mietz et al 1992; Dyson et al 1989; Sherman et al 1996; Phelps et al 1992]. Because HPV infection does not always lead to cervical cancer, other genetic alterations must also play a role in tumor development. Moreover, epidemiological data and experimental studies demonstrate clearly that infection *per se* does not suffice to induce malignancy. Additional genetic alterations seem to be required for their development and progression.

Cervical carcinomas develop as a result of multiple genetic alterations, and specific alterations lead to specific clinical behavior. However, the effect of such alterations on the occurrence and progression of preinvasive cervical cancer remains unknown. A loss of heterozygosity (LOH), which points to a role for tumor suppressor genes (TSGs), oncogene amplification, and point mutations, are all thought to be involved, but there is as yet no complete picture of the relative roles for each of these genetic changes in patients with cervical carcinomas. To play a role in tumorigenesis, both copies of a TSG must be inactivated. The loss of one allele in a chromosome region may point to the presence of a TSG in that region. Several studies have shown that LOH at specific chromosomal sites is frequently associated with the recurrence of various cancers, *e.g.*, 13q14.3 in oral carcinoma [Ogawara et al 1998], 10q in human lung cancer [Petersen et al 1998], and 11p15 in breast cancer [Karnik et al 1998]. Although cytogenetic studies of cervical cancer are relatively few, they have revealed frequent, nonrandom chromosomal changes [Atkin et al 1990]. Studies of LOH in patients with cervical carcinoma have also reported a high frequency of allelic deletions affecting 3p [Jones et al 1992; Kohno et al 1993], 5p [Mitra et al 1995], 17p [Jones et al 1994; Mitra et al 1994; Mullokandov et al 1996; Harima et al 1999], and 18q [Kersemaekers et al 1998b]. A LOH on chromosome 6p has also been reported in patients with cervical carcinoma Mitra et al 1994; Mullokandov et al 1996; Rader et al 1996; Kersemaeker et al 1998a]. However, the importance of LOH on chromosome 6p in the recurrence of cervical cancer after radiotherapy remains unknown.

It has been shown previously that a significant number of invasive cervical cancers have nonrandom chromosomal losses in 3p, 6p, 10p, 11q, 2q, 6q, and 19q, thereby suggesting that genes involved in the suppression of tumor development or progression are located in these regions (Rader et al 1996). Among these genetic alterations, chromosome arm 6p is one of those most frequently involved in a loss of heterozygosity in patients with cervical carcinoma [Chatterjee et al 2001]. Cervical intraepithelial neoplasia III is considered the precursor lesion for invasive carcinoma of squamous type. In CIN III, the most frequent allelic lass was found in 3p and 6p. In addition, by using several derivatives of chromosome 10 for further fusion experiments, the chromosomal region responsible for senescence could be assigned to 10p14–p15. The potential significance of loss of gene function in this region is underlined by the high frequency (38.7%) of loss of heterozygosity in cervical cancers including early stage tumors [Poignee et al 2001].

On the other hand, the reason why not all human papillomavirus (HPV)-positive high-grade lesions of the cervix progress to cancer is not understood. Storey and colleagues [Storey 1998] showed that polymorphisms in codon 72 of p53 could determine the efficiency of HPV 16 or HPV 18 E6 in degrading p53 in vitro. These data were further supported by testing cervical cancer biopsy specimens from UK women, which showed a seven-fold enrichment of the arginine allele over the proline allele. Several groups have failed to confirm this result [Rosenthal 1998, Lanham 1998, Hayes 1998, Josefsson 1998]. In the latter reports no association of the p53 codon 72 arginine with cervical cancer was found. Moreover,the proportion with codon 72 arginine in the healthy controls was considerably higher in these reports than in the study of Storey and colleagues [Storey 1998]. However, in a recent article,Zahbe et al. [zahbe 1999] found that the p53 arginine polymorphism represents a potential risk for cervical cancer development, consistent with the concept proposed by Storey and colleagues. In other word, discrepancy between these studies still exists and the reason needs to be clarified.

The term atypical squamous cell of undetermined significance (ASCUS) describes a minor degree of nuclear pleomorphism limited to the basal layers of cervical epithelium in the absence of severe inflammation with associated normal mitoses, koilocytosis, or koilocytosis associated features. Cervical intraepithelial neoplasm grade 1 means a dysplasia lesion involves the several layers of cell at lower third squamous epithelium. From a corroborative study-- Laboratories enrolled in the College of American Pathologists Interlaboratory Comparison Program in Cervicovaginal Cytology,, it was found that median reporting rates for epithelial abnormalities were as follows: ASCUS, 4.5%; low-grade squamous intraepithelial lesion (low-grade SIL), 1.6% [Davey et al 2000].

The prognosis in cervical epithelial changes of uncertain significance was found to be similar to that of CIN1 [Heatley 2001]. Therefore, Women with ASCUS or CIN 1 who are followed up regularly are at low risk for development of invasive cancer [Rabb et al 1999; Melnikow et al 1998]. Assessment of cytologic follow-up for patients with atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions may be regarded as the standard recommended management [Alanen et al 1998]. Women with ASCUS or CIN 1 who are followed up regularly are at low risk for development of invasive cancer [Rabb et al 1999; Melnikow et al 1998].

Recently, it was found that 17-18% of ASCUS was stable or progressed. [Giudice et al 2000]. However, which factors that can predispose the lesion to progress are still unknown. Most recent international or domestic studies did not focus on this issue. In a recent report that studied 52 eligible patients having conizations or hysterectomies as their histological outcomes and were tested for loss of heterozygosity, it was found that use of loss of heterozygosity in at least one locus was useful for predicting presence of high-grade cervical neoplastic lesion in the conized specimen [Chang et al 2001]. In fact, predictors of persistent and regressed disease for ASCUS or CIN1 were not identified [Duggan et al 1998].

In our department, we do not do any surgical operations (such as conization) to the cases with ASCUS or CIN. It seems that our management is the same as that recommended recently worldwide [Alanen et al 1998]. Assessment of cytologic follow-up for patients with atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions may be regarded as the standard recommended management [Alanen et al 1998].

Because of that

1). women with ASCUS or CIN 1 who are followed up regularly are at low risk for development of invasive cancer [Rabb et al 1999; Melnikow et al 1998], and

2). prognosis in cervical epithelial changes of uncertain significance was found to be similar to that of CIN1 [Heatley 2001], and

3). assessment of cytologic follow-up for patients with atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions may be regarded as the standard recommended management [Alanen et al 1998], and early colposcopy is suggested to be the clinical policy to exlude high-grade lesions [Melnikow et al 1998], and

4). predictors of persistent and regressed disease for ASCUS or CIN1 were yet not identified [Duggan et al 1998], even after reviewing the domestical or international literatures up to the present time, we dare to decide to conduct this study.

In this prospective study, we intend to identify the methods which may be helpful to determine or predict the progress or regress of these early cervical lesions. The cases with ASCUS or CIN1 on Pap smear were followed up without doing conization or hysterectomy and will do a long term follow up. This study experients will include HPV status, and p53 polymorphisms, as well as the genetic alterations which will involve the loci that most frequently found to occur.

CIN introduction

Carcinoma of the uterine cervix is the second most common malignancy of women worldwide in both incidence and mortality[Pontén et al 1995; NIH]. Papanicolaou (Pap) smear screening is the most effective tool currently available for early detection, leading to a greater than 70% reduction in cervical cancer mortality since the test was introduced 50 years ago. However, the Pap smear is not a perfect test; it has a high false-negative rate (variously estimated at 2% to 40%), due to a combination of sampling error, processing artifacts and the nature of subjective interpretation[NIH, Larsen 1994]

HPV

The involvement of the HPV in the development of cervical cancer has been firmly established. Because HPV infection does not always lead to cervical cancer, other genetic alterations must also play a role in tumor development. Specific human papillomavirus (HPV) types appear to be necessary etiological factors for most cervical cancers. Nevertheless, additional genetic alterations seem to be required for their development and progression.

HPV and CIN

Certain human papillomavirus genotypes are etiological agents in the development of cervical carcinoma [Our Hansen 1991] HPV16 is the most frequently detected genotype in invasive cervical carcinoma as well as in cervical intraepithelial neoplasia (CIN) I-III. HPV 18 is also reported to be related with these lesions. Persistent HPV infection is a risk factor for the progression of a preinvasive lesion to invasive cancer. However, most high-risk HPV infections do not progress to cancer. Epidemiological studies have identified additional risk factors that may contribute to the development of cervical carcinoma. These include age at infection, smoking, hormonal factors, genetic predisposition, and immune response. [Schiffman 1993]

Thus,the tumour biology of cervical intraepithelial neoplasia and cervical cancer is unusual. A large variety of individually distinct forms crudely divided into slight, moderate, severe dysplasia

and carcinoma in situ exist. Virtually all contain genital human papillomavirus either as infectious virions or as episomal or integrated DNA. A proportion of infected women develop condyloma, precancer and subsequently, in a minority, invasive cancer. Risk of precancer is statistically related to infection with genital HPV, but differences in risk between populations with high and low prevalence of HPV are larger than expected from a direct correlation. Findings fit with HPV as a major risk factor, but other factors must also be operative.

High risk HPV, typically 16 or 18, is preferentially associated with high grade dysplasia and in situ cancer either because it increases risk of clonal progression to these forms or induces them de novo. Severe dysplasia, in situ and invasive cancer always present as monoclonal lesions. Spontaneous mutation rate and physicochemical carcinogens seem insufficient for the creation of a malignant phenotype in cells of the transformation zone. Currently HPV is the only strong candidate for such a feat. Any or all of the following mechanisms may play a role: overexpression of viral E6 and E7 genes, often triggered by disruption of control elements upon integration of viral DNA into the cellular genome, activity of specific (E6) configurations in certain HPV variants, inactivation of TP53 with decreased capacity for DNA repair and enhanced likelihood of accumulation of "transforming" mutations and viral integration at sites controlling function of cellular oncogenes and/or suppressor genes. Low risk types are almost always associated with squamous differentiation, HPV 16 usually also with squamous differentiation and HPV 18 with adenosquamous or adenomatous differentiation.

Definition of ASCUS and CIN1

The term atypical squamous cell of undetermined significance (ASCUS) describes a minor degree of nuclear pleomorphism limited to the basal layers of cervical epithelium in the absence of severe inflammation with associated normal mitoses, koilocytosis, or koilocytosis associated features. Cervical intraepithelial neoplase grade 1 (CIN1) means a dysplasia lesion involves the several layers of cell at lower third squamous epithelium. The new Bethesda System terminology has opened a series of problems about the ASCUS and Low-Grade Squamous Intraepithelial Lesion categories, particularly on their treatment and follow-up.

Interlab difference of ASCUS

However, there are interlaboratory or interobservor difference in the rate of ASCUS. In a recent study, a cytologic diagnosis of ASCUS were reviewed independently by 5 experienced pathologists [Grenko et al 2000]. Agreement was better performed for high-grade squamous intraepithelial lesions (HSIL) and low-grade squamous intraepithelial lesions (LSIL) compared to those for ASCUS. Intraobserver reproducibility in the interpretation performed for ASCUS ranged from poor to excellent. They conclude that variability in the interpretation of biopsy specimens plays an important role in the differences in rates of dysplasia reported for the follow-up of ASCUS. [Grenko et al 2000]

Incidence of ASCUS

In a corroborative study-- Laboratories enrolled in the College of American Pathologists Interlaboratory Comparison Program in Cervicovaginal Cytology, 768 laboratories returned the 1997 questionnaire focusing on atypical squamous cells of undetermined significance (ASCUS) and glandular cells of undetermined significance (AGUS).The study found median reporting rates for epithelial abnormalities were as follows: ASCUS, 4.5%; AGUS, 0.3%; low-grade squamous intraepithelial lesion, 1.6% [Davey et al 2000].

ASCUS and CIN1-- the same treatment

The optimal management of low grade Papanicolaou (Pap) smear abnormalities remains controversial.

In a recent study, it was found that the prognosis in cervical epithelial changes of uncertain significance is similar to that of cervical intraepithelial neoplasia grade 1 (CIN1) [Heatley 2001]. In his study, the slides from 128 women with low grade cervical abnormalities, accessioned consecutively, were reviewed. In 43 women the initial diagnosis of ASCUS was confirmed and in 30 women the initial diagnosis of cervical intraepithelial neoplasia grade 1 was confirmed. Comparison of follow up data from these 73 women revealed a similar prognosis in the two groups in terms of regression to normal, persistence of low grade disease, or progression to high grade CIN. Therefore, low grade cervical disease (ASCUS and CIN1) should be managed according to similar treatment protocols [Heatley 2001].

Assessment of cytologic follow-up for patients with atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions may be regarded as the standard recommended management. [Alanen et al 1998]

Long term follow-up of ASCUS

Until now, few studies have compared long-term follow-up and risk for invasive cancer in women with atypical squamous cells of undetermined significance (ASCUS). In a study by Raab et al [Rabb et al 1999], they conducted a 6-year review of pathology files for 651 women in whom ASCUS had been diagnosed in 1992. Data collected included patient demographics, follow-up diagnoses, time between follow-up examinations, and procedures performed. At follow-up, high-grade squamous intraepithelial lesions had developed in 9.0% of the women, and invasive cancer in none. Previous cervical history did not affect risk for an HSIL. Although the average time to first follow-up was 6.18 months, in 20.9% of the women the diagnosis of HSIL was not established until after 2.0 years. For individual pathologists, the percentage of HSILs ranged from 0% to 18.8%. Thus women with ASCUS who are followed up regularly are at low risk for development of invasive cancer [Rabb et al 1999].

Melinkow et al made a meta-analysis on the natural history of cervical squamous intraepithelial lesions to estimate rates of progression and regression without reatment [Melnikow et al 1998]. Eligible studies, representing 27,929 patients, were stratified according to entry cytologic findings. The following rates of progression to high-grade SIL at 24 months were found: ASCUS, 7.13% (95% confidence interval [CI] 0.8%,13.5%); low-grade SIL, 20.81% (6.08%, 35.55%); and high-grade SIL, 23.37% ,12.82%, 32.92%). The following rates of invasive cancer at 24 months were found: ASCUS, 0.25% (0%, 2.25%); low-grade SIL, 0.15% (0%, 0.71%); and high-grade SIL, 1.44% (0%, 3.95%). The following rates of regression to normal were found: ASCUS, 68.19% (57.51%, 78.86%); low-grade SIL, 47.39% (35.92%, 58.86%); and high-grade SIL, 35.03% (16.57%, 53.49%). Study heterogeneity was not explained by regression analysis of study level variables. Their findings for borderline and low-grade abnormal cervical cytologic results suggest a relatively low risk of invasive cervical cancer with observation up to 24 months and support the clinical policy of early colposcopy for high-grade lesions [Melnikow et al 1998].

ASCUS,CIN progress factors—unclear yet

Recently, it was found that 17-18% of ASCUS were stable or progressed. [Giudice et al 2000].

Which factors that can predispose the lesion to progress are still unknown.

LOH of cervix neoplasms

Cervical carcinomas develop as a result of multiple genetic alterations, and specific alterations lead to specific clinical behavior. However, the effect of such alterations on the recurrence of cervical cancer after radiotherapy remains unknown.

A loss of heterozygosity (LOH), which points to a role for tumor suppressor genes (TSGs), oncogene amplification, and point mutations, are all thought to be involved, but there is as yet no complete picture of the relative roles for each of these genetic changes in patients with cervical carcinomas. To play a role in tumorigenesis, both copies of a TSG must be inactivated. The loss of one allele in a chromosome region may point to the presence of a TSG in that region. Chromosome arm 6p is one of those most frequently involved in a loss of heterozygosity in patients with cervical carcinoma. [Harima et al 2000]

Microsatellite instability

Human cancers progress through the accumulation of clonal genetic changes. Loss of heterozygosity has been demonstrated in almost all tumors analyzed to date and is easily detected by PCR-based microsatellite analysis. It has been demonstrated that appropriately selected microsatellite loci are commonly altered in many cancers and can serve as clonal markers for their detection. [Mao et al 1994] Using a panel of 13 microsatellites, we were able to detect 95% of transitional cell carcinomas of the urinary bladder by analysis of urine DNA. [Mao et al 1996] Furthermore, using a similar panel, 90% of bladder recurrences were detected prospectively. [Steiner et al 1997] In two cases, molecular changes preceded the clinical diagnosis of cancer.

Microsatellite instability of cervical cancer

In search of potential tumor suppresser genes (TSG), many LOH studies have been performed on primary cervical carcinomas. As in many other tumor types, cervical carcinoma displays frequent LOH at several loci, including those encompassing known tumor suppressor genes (TSG) [Rader et al 1996; Jones et al 1997; Mitra et al 1994; Mullokandov et al 1996; Kersemaekers et al 1998a]. One group of investigators evaluated 53 untreated primary cervical carcinomas at 57 loci and 49 (92.5%) tumors showed losses in 1 to 13 chromosomal arms.[Mitra et al. 1994] In a study by Rader *et al.*,[Reader et al. 1996] 80% of the tumors harbored at least one locus with chromosomal loss. The most common losses in these studies occurred on chromosomal arms 3p, 5p, 6p and 11q and helped us select our initial panel of markers to screen primary tumors. Consistent with previous findings, we noted LOH in more than 30% of primary tumors at many loci. These LOH studies also suggested that certain chromosomal losses might be early events in cervical carcinogenesis. Microsatellite abnormalities have been found at 3p, 5p, 9p and 11q in HSIL or carcinoma *in situ* (CIS) lesions, accompanying the invasive carcinoma.[Wistuba et al. 1997; Chung et al. 1992; Evans et al 1998; Mitra et al. 1995] Although it is less common, LOH has also been found in some low-grade (L)SIL.[Wistuba et al. 1997] These early losses were also detected in smears with few dysplastic cells, consistent with our observations in urine DNA from bladder

cancer and saliva DNA from oral cancers.[Mao et al 1996; Steiner et al 1997; Spafford et al 2001] In addition, Mullokandov et al [Mullokandov et al 1996] found that Chromosome arms 1q, 2q, 3q, 4p, 4q, 5p, 5q, 6q, 7q, 8p, 8q, 11q, 13q, 16p, 18p, and 19p are involved in LOH in 20-33% of the cervical tumors. LOH was found to involve 19 chromosome arms in 20-43% of the tumors. Chromosome arms 6p, 3p, and 18q are most frequently involved in LOH in 43, 39, and 35% of the informative carcinomas, respectively. The respective regions involved are 6p21.1-23, 3p13-25.3, and 18q12.2-21.2. LOH is generally limited to specific band segments within these regions. Similar high incidences of LOH of the same 3p segments have been reported in cervical carcinomas from different parts of the world. The same 3p and 6p segments are involved in many types of common cancers, whereas 18q changes are less frequent in other cancers. In their study, 11p was involved in 16% of the tumors, and 11q was involved in 22%. Chromosome 17 alterations are found in more cancers than those of any other chromosome, frequently involving the p53 gene on 17p. LOH of 17p was found in 5 (15%) cervical tumors; 2 of these were HPV negative and expressed mutant p53. In such HPV-negative tumors, direct mutation of the wild-type p53 appears to replace the inactivation of the p53 product by oncogenic HPV types. Tumors with LOH at many loci were, on the average, at more advanced stages, as were tumors with mutant p53. The higher overall incidence of LOH in cervical carcinomas as compared to other cancers, and the diversity of LOH patterns found, suggest that different cervical carcinomas probably arise and/or progress, in part, because of the loss of function of different yet finite sets of tumorigenicity suppressor genes and genes that are involved in tumor progression and metastasis. Their findings also indicate that certain chromosome segments that are often altered in cervical carcinomas are also frequently altered in several other types of cancers. All these studies support the presence of large clonal patches of cells that appear phenotypically normal in organs that harbor cancer.

In cervical carcinoma, microsatellite instability is reported as a rare event. In most studies, RER^+ (MSI[+]) tumors were found in only 2% to 6% of cervical carcinomas. Highly selected larger repeats can display higher rates of MI in many cancers, unrelated to mismatch repair deficiency.[Mao et al 1994; Mao et al 1996; Steiner 1997; Sidransky 1991; Baker et al 1990; Xu et al 2000] These microsatellites have been particularly useful for clonal detection and therefore were used in our group of primary tumors. With these selected markers, 55% of the tumors showed instability at one or more markers. However, detection of LOH was more common than MI using a panel of nine markers in both tumor tissue and Pap smear DNA. In a study by Harima et al, heterozygosity on chromosome 6p21.2 has been found as a potential marker for recurrence after radiotherapy of human cervical cancer [Harima et al 2000].

Microsatellite analysis of Pap smear DNA showed high specificity and sensitivity, although the detection rate was somewhat lower than the conventional Pap smear test. Even though the detection rate of the conventional Pap smear is high, there are still false-negative cases that might be detected by molecular analysis. Moreover, the conventional Pap smear evaluation can potentially be improved by new technologies including preparations from liquid-based specimen collection and automated screening techniques. In this study, the number of microsatellite markers applied to each sample was limited because the amount of DNA extracted from Pap-slides was also limited. Clearly more DNA can be obtained from liquid cell suspensions, such as that used for human papillomavirus detection, and application of more microsatellite markers may increase the detection rate.[Steiner et al 1997] With the advent of microcapillary automation using fluorescence-based DNA technology, the use of a larger panel of microsatellites is no longer a barrier.[Wang et al 1997].

Recent reports

From a cohort of 498 women with minimally abnormal Papanicolaou test results including atypical squamous cells of undetermined significance and low-grade squamous intraepithelial lesion

who had documented repeated Papanicolaou and human papillomavirus tests. Chang et al studied 52 eligible patients having conizations or hysterectomies as their histologic outcomes were subjected to tests of loss of heterozygosity on a panel of 5 microsatellites (D3S1110, THRB, D3S1228, D6S291, D3S1289) within the deoxyribonucleic acid of exfoliated cervical epithelia [Chang et al 2001]. They had interesting findings: With the use of loss of heterozygosity in at least one locus for predicting high-grade cervical neoplastic lesion, the sensitivity, specificity, positive predictive value, and negative predictive value were 96.7%, 59.1%, 76.3%, and 92.9%, which were superior to those of the human papillomavirus test (80%, 59.1%, 72.7%, and 92.9%). As a triage for atypical squamous cells of undetermined significance, its sensitivity and negative predictive value were up to 100%.

In a recent report of a pilot study by Rha et al. [Rha et al. 2001], they found that microsatellite alterations detected in the Pap smear DNA were identical to those identified in seven paired primary tumors available for analysis. Moreover, they also found that this molecular approach detected genetic alterations in two cases apparently negative by cytologic examination. None (0/25) of the control patients displayed microsatellite alterations in paired Pap smears. They concluded that icrosatellite analysis of cervical cytologic samples may provide a complementary method to analyze suspicious but not diagnostic cytologic samples further. In this pilot study, they detected microsatellite alterations (LOH or MI) in 85% of Pap DNAs from invasive carcinoma and 63% from SIL. As in cytomorphologic examination, the lower detection rate for SIL may be explained by the presence of smaller patches of clonal cell population and/or sampling error in obtaining the cervical scrape specimen. These lower grade lesions may contribute fewer dysplastic cells during the Pap smear scrape. The microsatellite abnormalities detected in the Pap smear DNA were identical to those seen in primary tissues. In a single case (*ACTBP2*, patient CA1), LOH was detected in both the tumor and Pap smear DNA, whereas only a faint shift was found in the primary tumor DNA. The population of cells sampled by the Pap smear may have lost the shifted allele or, more probably, came from another region of the tumor, as previously described for bladder cancer [Steiner 1997]. The recent pilot study by Rha et al demonstrates the potential use of molecular analysis in Pap smear samples, especially when cytological diagnosis is inconclusive but clinical suspicion is high. Based on this pilot study, they think that further exploratory studies are needed to develop the optimal panel of microsatellites for Pap smear DNA screening.

In an effort to define which chromosomal losses are present in the precursor lesions, Rader et al identified CIN III lesions from 24 invasive cervical cancer treated by radical hysterectomy [Rader et al 1998]. Thirty-three CIN III associated with 22 squamous carcinomas and 2 adenocarcinomas were carefully microdissected from the paraffin-embedded sections. The whole genomic DNA from CIN III was amplified with short random primers. DNA from invasive cervical cancer, CIN III, and normal tissue was analyzed at the six chromosomal regions with polymorphic markers. Thirty-eight percent of hysterectomy specimens had loss of heterozygosity (LOH) in at least one of the CIN III lesions from each case. Loss occurred in 30% of cases in 3p14.1-12 (37% for associated invasive cervical cancer), 21% in 6p23 (33%), 14% in 2q33-37 (27%), 0 in 11q23.3 (33%), 4% in 19q13.4 (13%), and 0 in 6q21-23.3 (18%). These results suggest that mutations in 3p and 6p are important early in tumorigenesis, whereas 11q and 6q contain genes important later in tumor progression. They concluded that invasive and preinvasive cervical lesions appear to develop from multifocal genetic events since consistent losses do not occur within all precursor lesions in the same patient.

High-risk human papillomavirus types 16 and 18 are involved in the multistep process of cervical cancer. Transfection of normal keratinocytes with high-risk HPV-DNA generally gives rise to immortal cultures. This may be explained by the loss of senescence genes as a consequence of HPV-induced genetic instability. On the basis of the dominance of cellular senescence over immortality, fusion of normal keratinocytes with HPV-immortalized cells results in

complementation of these putative gene defects. In a previous study, it has been shown that that underrepresentation of chromosome 10 is a characteristic phenomenon during the early phase of immortalization. Here we show that introduction of a normal copy of chromosome 10 into HPV16-immortalized cells (HPKII) by Microcell-mediated chromosome transfer resulted in senescence of a significant number of hybrids. By using several derivatives of chromosome 10 for further fusion experiments, the chromosomal region responsible for senescence could be assigned to 10p14–p15. The potential significance of loss of gene function in this region is underlined by the high frequency (38.7%) of loss of heterozygosity in cervical cancers including early stage tumors [Poignee et al 2001].

We do not do cone or op for CIN1 or ASCUS

In our department, we do not do any surgical operations (such as conization) to the cases with ASCUS or CIN. It seems that our management is the same as that recommended recently worldwide. Assessment of cytologic follow-up for patients with atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions may be regarded as the standard recommended management [Alanen et al 1998]

Factors still unknown

Until now, which factors may alter the progress of CIN to invasive cancer are still not clear.

Bertelsen et al [1996] examinated the wide accepted thesis that HPV infection is common in cervical intraepithelial neoplasia and is responsible for its progression to grade 3. Their findings suggest that a further factor, a cocarcinogen, may be involved in progression to CIN 3, HPV being a common forerunner, providing a proliferative environment and thus favoring such an event [Bertelsen et al [1996].

In a results reported by Ngan et al, EGFR, c-erbB-2 and c-myc may be important proto-oncogenes in CIN and that antibodies or anti-genes targeted against them may alter the progress of CIN to invasive cancer was suggested [Ngan et al 1999]. In their study, the factors were evaluated by immunohistochemical staining of normal cervical stratified squamous epithelium and CIN.

In a study on natural history of CIN 1 lesions [Duggan et al 1998], a longitudinal study of 342 women referred for colposcopic examination of a CIN I detected by a screening Pap test, and classified by the colposcopic impression and Pap test at that exam as equal to or less than CIN 1 was designed to identify predictors of disease outcome. The cohort was comprised of 220 women who satisfactorily completed the study and whose disease was neither biopsied or treated at the initial examination. All had HPV DNA testing by PCR, and were followed with interval colposcopic examinations and repeat Pap tests for a limited time period. The initial HPV DNA status and a number of measured clinico-pathological and risk factor variables were analyzed to identify outcome predictors. All underwent a biopsy either at the conclusion of the study or because their disease was considered to have progressed during the follow up period. Biopsy confirmed progression to CIN II/III occurred in 41 (18.6%), persistence of CIN I/Condyloma in 41 (18.6%), and regression to <CIN I/Condyloma in 138 (62.7%). HPV DNA positivity and current, oral contraceptive use were the only independent predictors of progression when age at diagnosis, the number of follow up visits, and time to progression were controlled. Predictors of persistent and regressed disease were not identified [Duggan et al 1998].

In a study by Larson et al, genetic alterations accumulate during cervical tumorigenesis and indicate a common origin for multifocal lesions
[Larson et al 1997].

Domestic study on CIN in Taiwan

Domestically, there are many study on CIN. In a study by Sun et al., measurement of human papillomavirus viral load in prediction of histologic severity and size of squamous intraepithelial

lesions of uterine cervix [Sun et al 2001]. Chu et al performed allelotyping of three 3p markers (at 3p14, 3p22-24, and 3p25) on 22 LSILs and 15 HSILs microdissected from patients with multiple or uniform cervical lesions [Chu et al 1999]. Frequent and early allelic loss was noted (in 30% of LSILs and 50% of HSILs) at 3p14, which may harbor tumor suppressor genes involved in early stages of cervical carcinogenesis. A high frequency of microsatellite alteration was found in LSIL and HSIL but not in invasive cancer. In particular, the alteration was more frequently found in low-grade lesions in association with invasive cancers than in those associated with SILs [Chu et al 1999]. Liu et al studied FHIT (fragile histidine triad) gene, which was identified at chromosome 3p14.2, in cervical intraepithelial neoplasia. Thirty-five consecutive CIN lesions taken from conization specimens and 33 normal cervical epithelial tissues taken from hysterectomy for benign diseases were included in their study. RT-PCR and mmunohistochemical study were performed. They found that the normal-sized FHIT transcript was present robustly in all of the CIN lesions and the abnormal FHIT transcripts occurred with similar frequency and pattern in the CIN lesions and normal cervical tissues. They suggested that abnormal FHIT transcription might not be causal in the early process of cervical carcinogenesis [Liu et al 2001]. Chang et al. determined microsatellite alteration in dysplastic lesions and evaluated the feasibility of the use of deoxyribonucleic acid microsatellite alterations in cervical epithelia in the prediction of high-grade dysplasia and to compare it with a strategy based on human papillomavirus testing [Chang et al 2001]. Fifty-two eligible patients having conizations or hysterectomies as their histologic outcomes were subjected to tests of loss of heterozygosity on a panel of 5 microsatellites (D3S1110, THRB, D3S1228, D6S291, D3S1289) within the deoxyribonucleic acid of exfoliated cervical epithelia. With the use of loss of heterozygosity in at least one locus for predicting high-grade cervical neoplastic lesion, the sensitivity, specificity, positive predictive value, and negative predictive value were 96.7%, 59.1%, 76.3%, and 92.9%, which were superior to those of the human papillomavirus test (80%, 59.1%, 72.7%, and 92.9%)[Chang et al 2001]. Jain et al studied predictive value of human papillomavirus test following conization of the cervix uteri. In their study, a prospective analysis was undertaken on 79 cone biopsies of women with high-grade lesions (CIN III). HPV testing was performed on cervical smears before and after conization. They found that HPV testing is potentially an effective tool in predicting residual dysplasia after conization and could potentially assist in the decision between hysterectomy and conservative follow-up in women with CIN III [Jain et al 2001]. Lin et al also confirmed an excellent sensitivity and negative predictive value of human papillomavirus deoxyribonucleic acid testing after conization in predicting residual cervical neoplasia. A strategy of managing patients with grade 3 cervical intraepithelial neoplasia, based on postconization human papillomavirus deoxyribonucleic acid findings and endocervical curettage results, was thusproposed [Lin et al 2001]. Law et al. found that high prevalence of high grade squamous intraepithelial lesions and microinvasive carcinoma in women with a cytologic diagnosis of low grade squamous intraepithelial lesions. A high percentage of CIN 2/3 as well as microinvasive lesions will go unnoticed in the absence of colposcopic evaluation [Law et al 2001]. Although there are many studies on CIN or ASCUS internationally or domestically, the continuous problem of which factors may determine the progress or regress of these lesions still remains.

Our purpose

In our department, we do not do any surgical operations (such as conization) to the cases with ASCUS or CIN. It seems that our management is the same as that recommended recently worldwide. Assessment of cytologic follow-up for patients with atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions may be regarded as the standard recommended management [Alanen et al 1998]. The aim of this prospective study is to identify the methods which may determine or predict the progress or regress of early cervical

lesions. In this study plan The cases with ASCUS or CIN1 on Pap smear were followed up without doing conization or hysterectomy and will do a long term follow up. This study will include the genetic alterations, HPV status, and p53 polymorphisms. The genetic alterations will include the loci which most frequently found to occur. These laboratory work were assessed as predictive tests for persistence and progression of ASCUS of CIN1.

Materials and Methods :

Case recruitment

The candidates for this study were recruited from the women visiting our hospital who had a routine conventional cervical Papanicolaou smears. Woman whose Pap smear results revealed ASCUS or CIN1 were referred to do colposcopy. A cervical biopsy and/or endocervical curettage were performed if any apparent lesions are found. The cases that have not been diagnosed as having lesion as high grade intraepithelial lesions or more advanced lesions were included in the study. The samples were collected with a cervical swab from both the endocervical and exocervical areas. The samples were put into the tube containing 1x PBS until processing is performed.

Follow-up

Follow-up Pap smear, colposcopy, HPV, P53 polymorphism, LOH, MI at 6- month intervals.

DNA extraction

Smear samples were centrifuged at 4000g for 6 min and washed twice with PBS. Then they were processed including SDS/proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. Normal control DNA were obtained by venipuncture and isolation of lymphocytes DNA.

Normal control

Pap smears and peripheral blood from ~~25~~ patients without any precancerous or cancerous cervical lesions were obtained from the same hospital as normal controls.

Analysis for alleic loss and microsatellite instability

DNA derived from leukocytes, tumor and Pap smears was analyzed using a panel of microsatellite markers on different chromosomes (Research Genetics, Huntsville, AL). One primer of each marker pair were end-labeled with [γ-^{32}P] ATP (Amersham, Arlington Heights, IL) using T4-polynucleotide kinase (New England Biolabs, Beverly, MA). Genomic DNA (50 ng) were subjected to 35 cycles of PCR at a denaturing temperature of 95-C for 30 sec, followed by varying annealing temperatures ranging from 50 to 60-C for 1 min, an extension at 70°C for 1 min and a final extension step at 70 °C for 5 min on a Hybaid thermocycler (Hybaid, Teddington, UK). PCR products will thus then be separated in denaturing 7% polyacrylamide -urea -formamide gels and exposed to film from 4 to 48 hr as described. On performing the stepdown PCR, we will use AmpliTaq Gold™ (Perkin-Elmer, Branchburg, NJ) with GeneAmp® 10?PCR Gold Buffer instead of our standard conditions.

For informative cases, allelic loss were documented if one allele is significantly decreased (>50%) in the Pap smear DNA compared with the same allele in the normal (lymphocyte) DNA. MI

were described if an additional band representing a change in repeat number was noted in the lesion or Pap smear compared with normal.

HPV detection and typing

Human papilloma virus status will also be studied . All smear specimens will tested for the presence and type of HPV DNA by using a degenrate PCR-based method with a panel of oligonuleotide primers located within the highly conserved L1 open reading frame of the HPV genome. Ampliphied PCR products will then be sequenced.Basically, two different consensus primer sets A and B were used for PCR amplification to detect HPV DNA. These primer sets are at least able to amplify sequences of HPV types 6, 11, 18, 31, 33, 39, 45, 51, and 56 [Toshikawa 1991; Fujinaga 1991]. Each 25 μl of PCR mixture contains 2 μl of sample DNA solution containing 25-250 ng of genomic DNA fragment, 0.35 μM primers, 2.5mM MgCl2, 0.25 mN dNTP, 2.5 μl of suppled PCR buffer and 0.5 U of Tag polymerase (Boehringer Mannheim). PCR was carried out as follows. The PCR product were electrophoresed on 2% agarose gels stained with ehidium bromide and viewed under UV light. Samples identified as HPV-positive with these sconsensus primers were then HPV genotyped with type 16 and 18 specific primers [De Roda Husman 1995].

HPV consensus A (forward) 5'-TTTTCACCCATCTACAGTCCCCCTTG-3'
HPV consensus A (reversed) 5'-TACCCTAAATACTCTGTATTG-3'
HPV consensus B (forward) 5'-TGTCAAAAACCGTTGTGTCC-3'
HPV consensus B (reversed) 5'-TCTGAGTCGCTTAATTGCTC-3'
HPV 16 specific (forward) 5'-TACACGCAGTACAAATATGT-3'
HPV 16 specific (reversed) 5'-ATTCCTCCCCATGTCGTAGG-3'
HPV 18 specific (forward) 5'-CACTCGCAGTACCAATTTAA-3'
HPV 18 specific (reversed) 5'-ATTCCTCAACATGTCTGCTA-3'

PCR amplification of p53 polymorphic sequences

To detect p53 polymorphism at condom 72, we will use allele-specific PCR and PCR-restriciton fragment length polymorphism. The plasmid pBR322 digested with MspI were used as size marker. Fifteen microliters of extracted DNA were used in a 50 μl PCR containing 50 mM KCL, 10mM Tris-HCl(pH 8.3)m 200 μM of each dNTP, 1.5 mM MgCl2, 1U Tag DNA polymerase (Gibco BRL) and 50 pmol of each primer.The following primer pairs were used based on those described by Storey et al., using p53/Arg- amplifying a 137-bp fragment from the arginine allele; and Pro+/p53- amplifying a 206-bp fragment from the proline allele.

P53+, 5'-GTCCCCCTTGCCGTCCCA-3'
Arg-, 5'-CTGGTGCAGGGGCCACGC-3'
Pro+, 5'-GCCAGAGGCTGCTCCCCC-3'
P53-, 5'-GGAAGCCAGCCCCTCAGG-3'

Samples were subjected to an initial denaturation step at 95oC for 2 min, followed by 40 cycles of denaturation at 94oC for 45 sec, annealing at 60oC for 45 sed, extension at 72oC for 30

sec and a final period of extension at 72oC for 5 min. Both amplicons (10 μl) will undergon electrophoresis in a 9% polyacrylamide gel containing 5% glycerol and a 35-75% denaturing fradient (7M urea/40% deionised formamide) at a 150 V for 7.5 hours at 59°C and were stained with ethidium bromide.

The polymorphism were confirmed in each case by amplification across the polymorphic site using primers p53+ and p53-, followed by restriction enzyme digestion with 20 U of the enzyme Bsh1236I (Stratagne, Cambridge).The recognition site is present only in the arginine-encoding allele [De La Calle-Martin 1990]. From this step, as a result, the arginine allele were identified by the presence of 2 fragments of 120 and 187 bp and the proline allele by a single frgment of 307 bp. Heterzygous samples will show all 3 fragments. CaSki cells, which are heterozygous for the polymorphism [Storey 1998], were used as a positive control in PCR for digestion.

To evaluate the relationship between progressiveness of ASCUS or CIN1 with presence or status of LOH, microsatellite instability, HPV, p53 polymorphisms a, we will divide the cases into those with progress and non-progress. Their changes between the two groups were compared.

Results and Discussion :

In this study, our original planning is that will finish this by three years. However, the Council only provide us for studying one year. Therefore, up to now, not all the original purpose has been finished. We are now still trying to finish our planning although the financial support has been limited. The confined supply from the government has now resulted in severe problem for my academic study, especially when the project of this year are not promitted. It is unfair for an university hospital staff.

Human papillomavirus typing and DNA content measurements may delineate a distinct group of ASCUS. Our preliminary data suggest that ASCUS cases with high-risk HPV positivity and with rare cells with abnormally high DNA content represent similar biologic features as high-grade SIL and are at elevated risk to develop cancer.

The distribution of p53 genotypes was not significantly different in all study groups (HPV positive vs HPV negative and cases vs controls comparisons). Homozygosity for Arg/Arg was not associated with increased risk for cervical cancer. We find no evidence for any association between homozygosity for p53 arginine with either cervical dysplasia, cervical carcinoma or HPV infection in our population.

From the eligible patients having conizations or hysterectomies as their histologic outcomes were subjected to tests of loss of heterozygosity on a panel of 5 microsatellites (D3S1110, THRB, D3S1228, D6S291, D3S1289) within the deoxyribonucleic acid of exfoliated cervical epithelia. These genetic alterations were analyzed through fluorescence polymerase chain reaction by comparison of allele ratios of exfoliated cells with those of normal control tissue. With the use of loss of heterozygosity in at least one locus for predicting high-grade cervical neoplastic lesion, the

results were analysed. As a triage for atypical squamous cells of undetermined significance, its sensitivity seems to be good. The promising results on determining microsatellite alteration in dysplastic lesions might imply that it is possible to detect the earliest changes by potential molecular markers with exfoliated cervical epithelial cells.

The conclusion from our present data seems do not support the hypothesis that this p53 polymorphism is involved in the development of high-grade squamous cervical disease in this population.

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實驗數據參考(一)

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SUBJECT

DATE 2002/9/26

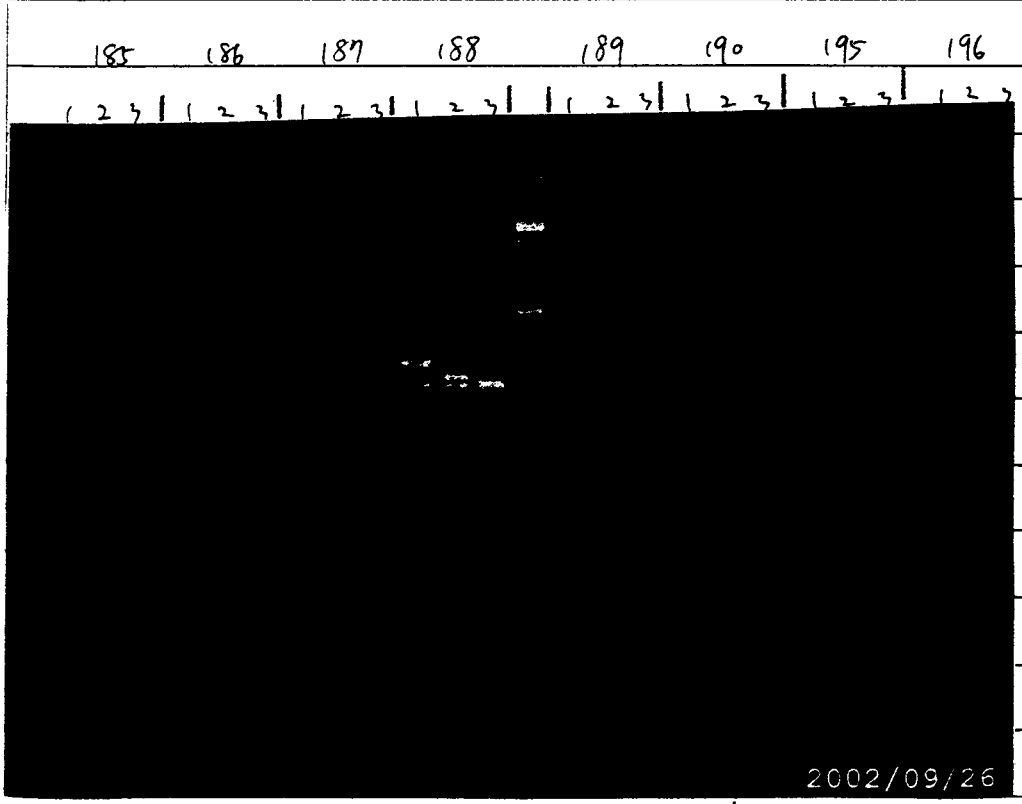
RFLP HPV 185~190 195~196

HPV - RFLP

* PCR product	NEB 1~3	Rsa I / Hae III / Dde I	$dd H_2O$	Total
5λ	1.0λ	0.1λ	3.9λ	10λ

Temp: 37°C ~ overnight

1. Rsa I
2. Hae III
3. Dde I



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SIGNATURE OF INVESTIGATOR DATE

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實驗數據參考(二)

SUBJECT

DATE 2002/10/30

RFLP p53

189 ~ 200

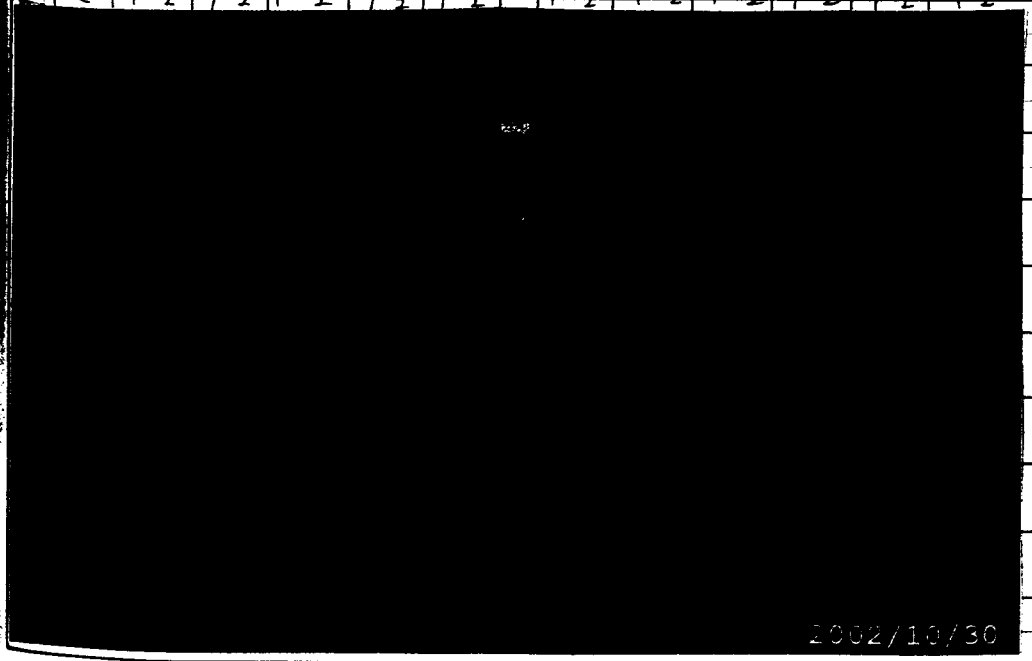
P53 - RFLP

* PCR product	NEB 4	SmaI / KspI	ddH2O	Total
5λ	1.0λ	0.1λ	3.9λ	10λ

Temp: 37°C ~ overnight

1. p53 SmaI
2. p53 KspI

189 190 191 192 193 194 195 196 197 198 199 200



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