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Genetic Instability as Biomarker during Multistep Tumorigenesis in Cervical Cancer

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Background: Cervical cancer is the second most common malignancy in women worldwide. Chemoprevention of cervical cancer is highly desirable to reduce incidence and prevalence of cervical cancer. Biomarkers for chemoprevention are essential to develop successful chemoprevention trials.

Purpose: to better understand the field cancerization and multistep tumorigenesis process in cervical intraepithelial neoplasia and invasive cancer of the cervix, we examined chromosome 9 polysomy in tissues that changed from histologically normal epithelium through cervical intraepithelial neoplasia to invasive cancer of the cervix.

Methods: 37 specimens from the same number of patients were studied. These specimens show transition from normal to CIN-3 - invasive cancer. Hybridization with DNA probes specific for the centromeric region of chromosome 9 was performed. A pathologist marked each pathological area. At least 200 nuclei were scored for each defined histological area for number of chromosomes. A chromosome index was obtained dividing the total number of signal spots by the number of nuclei analyzed. Differences for each specific pathological entity means were analyzed.

Results: An increase in the normalized chromosome index was present as the tissue progressed from normal to cervical intraepithelial neoplasia to invasive carcinoma. The frequency of cells with polysomy increased as the tissues progressed. Genotypic abnormalities adjacent to the tumor were present.

Conclusions: The determination of the degree of accumulated genetic abnormalities in cervical tissue may be useful for identifying individuals with high risk of progression to cervical cancer. This parameter may be a biomarker as an intermediate end-point in chemoprevention trials.

Key Words: Cervical cancer, Biomarkers, Genetic instability, Tumorigenesis, Chemopreventio

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INTRODUCTION

Cervical cancer is the second most common malignancy in women worldwide, accounting for 15% of all cancers diagnosed in women. The Surveillance epidemiology and end results (SEER) program estimates that in 1995, 15,800 women were diagnosed with invasive cervical cancer and 4,800 women died from invasive cervical cancer in the Unites States.[1] Cervical cancer remains a significant problem and the number one cause of death in developing countries. Despite an understanding of the epidemiologic risk factors, the screening Papanicolau smear and advances in cancer therapy, overall survival remains 40%.[2] New strategies based on the clinical and molecular aspects of cervical carcinogenesis are desperately needed. Chemoprevention of precancerous lesions is one such approach.[3]

The association between cervical neoplasia and sexual activity is well established[4,5] and current studies have identified the human papillomavirus (HPV) as the most important factor responsible for this association. HPV is considered a causal agent in cervical neoplasia.

The rational design of novel chemopreventive strategies requires an understanding of the fundamental event of tumor development. Chemoprevention trials would also benefit greatly by the elucidation of markers with which to identify individuals at highest risk and to monitor the efficacy of the chemopreventive agents in reversing or inhibiting tumorigenesis.[6] Tumorigenesis has long been thought to be a multistep process,[7] however, only recently has it become possible to identify the molecular events that underlie the initiation and progression of human tumors.[8,9] Recent studies in several human tumor systems have suggested that tumorigenesis is a multistep process, driven by an accumulation of genetic alteration, resulting in dysregulation of proliferation and differentiation and in cell loss.[10] Thus, potential biomarkers might include indicators of the degree of generalized and specific genetic change as well as the degree of cellular dysregulation in the tissue at risk for tumor development.[11]

One potential marker for the tumorigenesis process is the degree of genetic change in the tissue at risk. While a variety of cytogenetic changes have been described for head and neck tumors,[12~14] a comprehensive list of specific genetic changes has been limited by impediments common to solid tumor cytogenetic studies, i.e., the low frequency of mitotic figures from direct preparations, suboptimal chromosome preparations and significant complexity of cytogenetic changes.[15] Identification of karyotypic changes in premalignant lesions is technically even more difficult with conventional cytogenetic procedures and has seldom been reported.[16~18] Moreover, the spatial cellular distribution of genetic changes in premalignant and malignant lesions cannot be defined by conventional cytogenetic techniques because single cell preparations are required. Powerful molecular tools have recently become available for dissecting the multistep carcinogenic process. Molecular studies may uncover the key genotypic alterations that lead to the development of malignant clones. This work may also identify molecular markers of specific stages of multistep carcinogenesis which may become useful for establishing valid intermediate end points for chemoprevention trials. While a variety of clinical observation has suggested that cervical tumorigenesis involves a multistep process occuring in a field exposed to carcinogens, it was of interest to determine whether these processes could be correlated with genetic events occuring in the affected

tissue regions.

In this study, we examined chromosome 9 polysomy in tissues that transition from histologically normal epithelium through cervical intraepithelial neoplasia to invasive cancer of the cervix.

MATERIALS and METHODS

1) Tissue materials

Formalin fixed, paraffin-embedded tumor specimens were obtained from 250 patients with cervical intraepithelial neoplasia and invasive cancer of the cervix who underwent conization at the University of Texas M.D.Anderson Cancer Center between 1988 to 1994. From these 250, 37 cases showing transititon from none to CIN-3- invasive carcinoma were chosen. Inappropriate specimens for analysis, such as tangentially cut or degenerated, were excluded. All specimens and hematoxylin-eosin stained histological slides were reviewed by one pathologist to identify normal, cervical intraepithelial neoplasia 1,2,3 and invasive cancer areas. 4 um thick sections were mounted on aminoalkylsilane-coated slides

2) Chromosome in situ hybridization

Prehybridization Procedure: The specimens were incubated at 65°C overnight. Subsequently, the sections were dewaxed by immersing them two times in xylene for 15 min.each, followed by treatment with 100% ethanol, two times for 10 min. each. The slides were incubated at 80C for one hour and then treated with 100 ul of 10 mg/ml RNase in 2X SSC at 37°C for 30 minutes. 150 to 200 ul of 0.4% pepsin (Sigma) in 0.2 N HCl was applied to the slides and coverslips were applied. The slides were placed at 4°C for 15 min. to allow the pepsin to diffuse evenly throughout the tissue sections and then incubated at 37°C for 15-17 min. to allow protein digestion. After three washes with deionized water for 3 min, each was followed by dehydration through 70%, 90% and 100% ethanol. The specimens were placed in acetone for 2 min. at room temperature and then air dried. Endogenous peroxidase activity was blocked by dipping the samples in 10% H2O2 in methanol for 5 min.

Hybridization procedure: Biotinylated -satellite DNA probes specific for the centromeric region of chromosome 9 (Oncor.) were applied. The hybridization solution was composed of 60% formamide in 2X SSC, 5% dextran sulfate, 1 mg/ml salmon sperm DNA and 0.8 ng/ul biotinylated DNA probes. Thirty ul of the hybridization solution were applied to each section and covered with 22×22 mm coverslip, which was then sealed with rubber cement. The probe and target DNA were denatured together at 95°C for 6 min and incubated at 37°C overnight in a sealed wet chamber.

Detection of Hybridized DNA probes: After overnight incubation, the coverslips were removed and the slides were washed in 50% formamide, 1X SSC (pH. 7.0) for 15 min.twice at room temperature followed by three 10 min. washes in 0.1X SSC at 37°C. 100 ul of 5 ug/ul avidine (Vector Laboratories, Inc., Burlingame, CA) in 3% bovine serum albumine in PBS were added and the slides were incubated at 37°C for 30 min. in moist chamber. After being washed three times in PBD, 100 ul of 5 ug/ul biotinylated anti-avidine D (Vector Laboratories, Inc.) in 3% bovine serum albumin in PBS were applied to the slides and incubated at 37°C for 30 min. These steps were repeated twice to amplify the signals. 100 ul of avidine-biotin peroxidase complex solution (Vectastain ABC kit; Vector Laboratories, Inc.) were added and the slides were incubated at 37°C for 30 min. After being washed three times with PBD & PBS, the hybridized slides were stained with a 50 ml 1X PBS solution containing 50 mg diaminobenzidine tetrahydro-

chloride (Sigma), 35 mg of NiCl2 and 12 ul of 30% H2O2 at room temperature for 2-3 min. to allow signal development. The slides were then successively washed in 1X PBS for 5 min., rinsed in deionized running water for 10 min., air dried and counterstained with Giemsa stain for 30 sec. The sections were then mounted in Eukitt (Calibrated Instruments, Inc. Hawthorn, NY) and examined under a light microscope.

3) Analysis of chromosome copy number

Areas for analysis were selected by the pathologist by comparing the hybridized slides to a corresponding hematoxylin-eosin stained adjacent section. Since the centromeric region of a chromosome occupies only a small region of the interphase nucleus, the hybridized signal appears as small dark spots. At least 200 nuclei were scored in each defined histological area.

The following scoring criteria (19) were applied for the ISH signals: (a) nuclei should not be covered by cytoplasmic materials; (b) nuclei should not overlap; (c) signal intensity should be more or less of the same homogeneous staining intensity; (d) minor hybridization spots, which can be recognized by a smaller size and lower intensity should be excluded; (e) signals may only be counted when completely seperated from each other; and (f) paired or closely opposed spots should be counted as one signal.

The total number of signal spots was divided by the number of nuclei analyzed to obtain a chromosome index. Because of sectioning artifacts and the fact that hybridization efficiency may vary slightly from one experiment to another, normal diploid lymphocytes present in the same section were used as internal controls. Thus, it was felt that lymphocytes could also serve as quantitative internal controls. By dividing the chromosome index of the epithelial cells by that of the lymphocytes in the same section, a normalized chromosome index was obtained. Polysomy index was measured by the percentage of the cells which have more than three signals. Magiscan II image analysis system (Joyce-Loebl, Ltd., Dukesway, England) was used to examined the difference in the distribution of chromosome copy number of nucleus.

4) Statistical analysis

Analysis of the data was accomplished using Excel, Sigma-Plot, Cricket graph, and SPSS. The level of significance was set at p-value less than 0.05. Data from each disease category was analyzed independent of the patient. Further analysis, each patient as a whole was performed using the same software packages.

RESULTS

1) Patients and tissue characteristics

Of the 37 cases chosen, 26 exhibited histologically normal adjacent squamous epithelium, 19 CIN I, 18 CIN II, 24 CIN III and 7 invasive carcinoma of the cervix. All of the tumor specimens contained infiltrating small lymphocytes, which served as internal controls for hybridization efficiency and sectioning artifacts.

2) Genetic instability and histologic characteristics

Positive chromosome signals after non-isotopic in-situ hybridization and immunohistochemical detection appear as dark dots on the interphase nuclei in the tissue section. As illustrated in Fig. 1, epithelial cells of normal, CIN 1 and CIN2 showed 0, 1, 2, 3, signals/nucleus and epithelial cells of CIN 3 showed 0, 1, 2, 3, 4 signals/nucleus for chromosome 9. Invasive carcinoma showed 0, 1, 2, 3, 4, 5 signals/nucleus for chromosome 9.

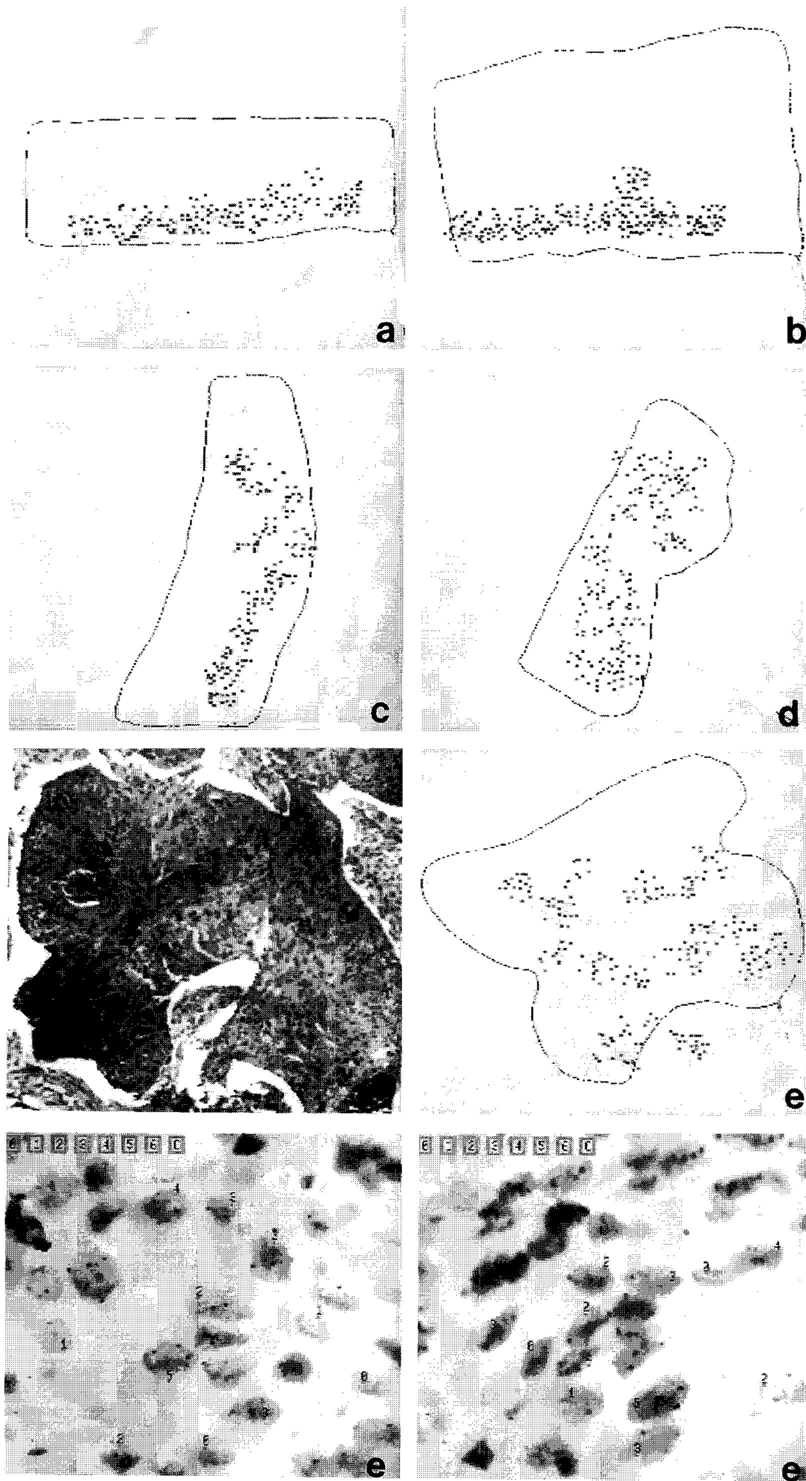

Fig. 1. Chromosome 9 ISH signals in CIN and invasive carcinoma of the cervix.: (a) normal, (b) CIN 1, (c) CIN 2, (d) CIN 3, (e) carcinoma.

Table 1. Genetic instability in CIN and invasive carcinoma of cervix

	No.of patients	N.C.I.* (mean±S.D.)	Polysomy index(%) (mean±S.D.)
Lymphocyte	9	1.03±0.04	0.97±0.54
Normal	26	1.06±0.06	3.15±1.03
CIN 1	19	1.11±0.10	4.64±2.13
CIN 2	18	1.23±0.12	8.00±3.37
CIN 3	24	1.36±0.11	12.19±4.47
Invasive carcinoma	7	1.61±0.11	21.36±3.76

*N.C.I.: normalized chromosome indices

For whole diploid cells, one would expect to see two signals. However, 2 or fewer signal/nucleus were observed here because 4 um thick sections produced truncated nuclei.

3) Increased normalized chromosome index (NCI) during multistep carcinogenesis

As shown in Table 1, there was a general trend toward increased normalized chromosome indices as the tissue progressed from normal through cervical intraepithelial neoplasia to carcinoma of the cervix. The mean normalized chromosome index in adjacent normal tissue showed 1.06. The mean normalized chromosome index of CIN 1, CIN 2 and CIN 3 were 1.11, 1.23 and 1.36 respectively. In contrast, invasive carcinoma showed 1.61 of mean normalized chromosome index for chromosome 9.

However, there was a considerable range of values with each histological characteristic such that a significant difference from normal epithelium was only observed as tissues progressed to CIN 1 ($p<0.04$), CIN 2 ($p<0.001$), CIN 3 ($p<0.001$) and to invasive carcinoma ($p<0.001$) (Fig. 2).

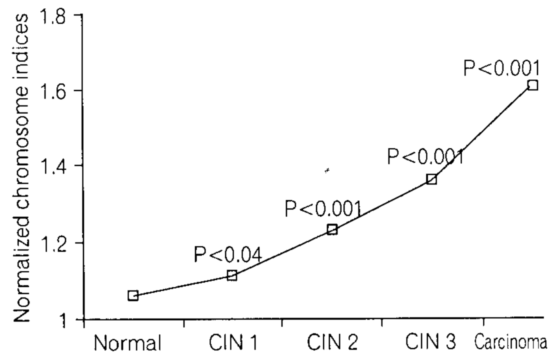

Fig. 2. Normalized chromosone indices for normal, CIN 1, CIN 2, CIN 3, and invasive carcinoma of the cervix.

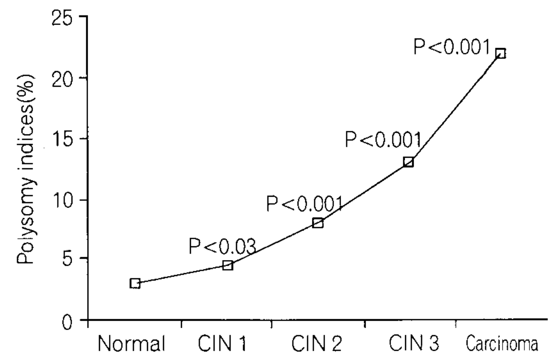

Fig. 3. Polysomy indices for normal, CIN I, CIN 2, CIN 3, and Invasive carcinoma of the cervix.

4) Polysomy index

To better detect genetic events in the tumor field, we determined the fraction of cases that exhibited cells with 3 or more copies of a chromosome/cell. If tumorigenesis of cervix is a multistep process resulting from an accumulation of genetic changes, one might expect to observe an incresed of chromosome polysomy with histological progression from normal to malignant tissue. The fact that these observations represented somewhat rare events in adjacent normal tissue is underscored by the finding that the mean fraction of cells exhibiting 3 or more copies was 3.15%. Nevertheless, this is different

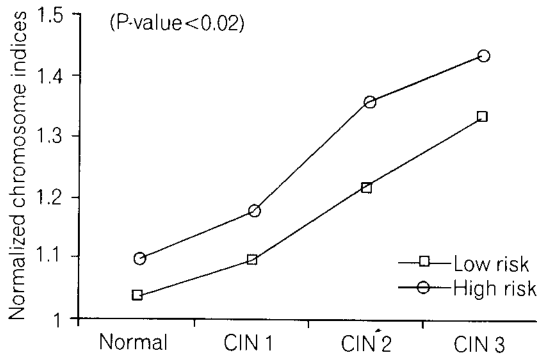

Fig. 4. Normalized chromosome index in low risk and high risk of premalignant lesions of the cervix.

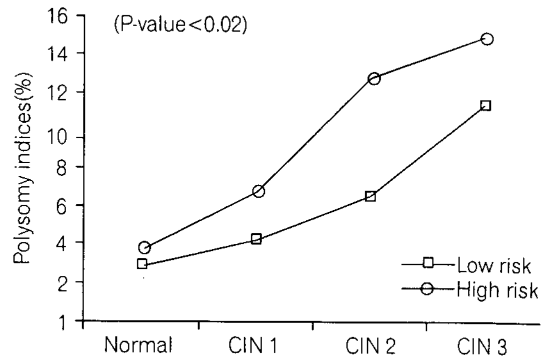

Fig. 5. Polysomy indices in low risk and high risk of premalignant lesions of the cervix.

from 0.97% for normal lymphocytes in tumor region. The frequencies of cells with 3 or more copies of CIN 1, CIN 2 and CIN 3 were 4.64% (range, 1.7～9.4%), 8.00% (range, 2.4～14.2%) and 12.19% (range, 5.7～20.0%) respectively. In contrast, invasive carcinoma showed mean of 21.36% (range, 16.3～26.3%) cells showing 3 or more copies/cell for chromosome 9 (Table 1).

5) When trying to test the hypothesis that high instability predicts the development of invasion, those cases which have invasion had higher mean - polysomy indices and mean-normalized chromosome index that those cases which did not have invasion (p=0.02) (Fig. 4 and 5). Premalignant lesions with higher polysomy index and normalized chromosome index are more likely to develop cancer.

DISCUSSION

The natural history of cervical intraepithelial neoplasia shows regression of CIN 1 in 60% of cases, persistence in 30%, progression to CIN 3 in 10% and progression to invasion in 1%. The corresponding approximations for CIN 2 are 40%, 40%, 20% and 5% respectively. The likehood of CIN 3 regressing is 33% and progressing to invasion greater than 12%.[20] Nasiell et al[21] reported a 62% regression rate for CIN 1 lesions in a large prospective study. Progression to either CIN 3 or invasive carcinoma occured in 16%, with an average time to progression of 48 months. In an earlier study on CIN 2 lesions, the subgroup of patients who did not undergo a biopsy for diagnosis had a 50% regression and 35% progression rate with an average time to progression of 51 months.[22] It is generally agreed upon that most patient with CIN 3 will eventually develop invasive cancer. Varied estimates have been made regarding the duration it takes carcinoma in situ to progress to invasive carcinoma, with a range of 3 to 10 years reported by Barron et al.[23] Thus, markers of progression are highly sought.

The multi-step process from HPV infection to carcinogenesis is not yet completely understood. HPV genetic sequences have been observed to be integrated into the host genome just as the cell develop invasive properties.[24] It is known that the E6 protein produced by high risk HPV types 16 and 18 can complex the p53 protein and results in the same functional consequence as a p53 gene mutation.[25,26] In contrast to this, E6 protein expression from the low risk HPV 6 does not produce any such effect. The E7 protein of HPV 16 was also

shown to bind to the p105-Rb protein encoded by the retinoblastoma gene (Rb1).[27] Both p53 and the pRb proteins participate in the G1-S cell cycle checkpoint that normally causes cells with DNA damage to undergo either cell arrest at G1 or apoptosis.[28] The E6 and E7 oncoproteins produced by HPV 16 have been shown to undermine this cell cycle checkpoint by causing a decrease in p53 and pRb protein levels respectively.[29,30] The alteration of the G1-S checkpoint leads to the inappropriate survival of genetically damaged cells and may thus be a step in the development of malignancy. Various cofactors are probably necessary for the complete progression towards carcinogenesis. Somatic mutation of the p53 gene may also be present in the cervical carcinoma but are uncommon.[31]

In the multistep carcinogenesis, epithelial cancer appears to develop in a recognizable and predictable series of steps. Epithelial carcinogenesis is conceptually divided into three phases: initiation, promotion and progression. In initiation, a carcinogen or procarcinogen interact with DNA to produce a fixed stable genetics event after DNA replication. In promotion, which is defined as the period between initiation and the appearance of a premalignant lesion, the initiated cells proliferate. In progression is the phase between a premalignant lesion and the development of invasive cancer.[32,33] This concept has recently been analyzed in molecular studies which have identified a continuum of accumulated specific genomic alterations that take place in the clonal evolution of epithelial neoplasia. The molecular genetic basis of the multistep process in human is most apparent in the progression of adenoma-carcinoma in the colon.[10]

In the field of carcinogenesis, it was proposed by Slaughter et al[33] that large areas of an epithelial surface or "field" are exposed to enviromental carcinogens and are at risk to develop cancer. This concept proposes that multiple neoplastic lesions of independent origin occuring within an epithelial field can result from repeated exposure to a single carcinogen. According to the field carcinogenesis theory, primary tumor and related second primary tumor result from progression of commonly initiated although genetically different premalignant lesions. Recent molecular genetic studies of histologically normal and premalignant epithelia from high-risk subjects and of malignant aerodigestive tract epithelia strongly support the field carcinogenesis concept. These studies suggest that multiple, genetically distinct lesions occur throughout the aerodigestive tracts of high risk subjects and cancer patient. The field carcinogenesis concept suggests that separate primary cancers that develop in the tissue region exposed to the same carcinogen would arise as independent clones with similar but unique molecular genetic alterations.[34]

The genetic determinism of cancer must be considered as a complex process affecting multiple structural and functional levels of the genome, incompatible with a simple oncogenic gene(s) activation or derepression model. Carcinogenesis is above all a network of interacting phenomena leading to lethal autonomous and autoamplified tumor cell clonal progression and diversification. Genetic instability is the obvious major driving force of this on-going expansion, and the role of chromosomal changes appears to be at least permissive and conditional.[35] Multiple types of genetic changes or events (gene mutation, deletion, amplication, translocation and mitotic recombination) occur early in carcinogenesis and continue to drive it throughout promotion and progression. The clonal evolution of neoplasm in association with genetic changes begins early in the carcinogenic process.[36] Many cytogenetic studies in solid tumors report highly variable karyotypes. In some cases, it is difficult to identify

the common chromosome changes associated with tumor development. These complex karyotypic patterns were previously ascrived to karyotypic instability once the tumor had developed.[37] In other cases, the complex karyotypic patterns observed were proposed to be the result of tumor heterogeneity or multiple tumor in the resected tissue.[38~40]

Recently, it has been shown that the technique of in situ hybridization (ISH) using chromosome-specific probes might be useful in this regard. This technique involves the use of DNA probes that recognize either chromosome-specific repetitive target sequences or sequences along the whole chromosome length or chromosome segments. Using this method, it has became possible to localize individual chromosomes in both metaphase plates and interphase nuclei, since chromosomes and chromosome regions appear to occupy domains within the nucleus.[41~43] ISH has been adapted for use on formalin-fixed, paraffin embedded tissue sections using nonisotopic, chromosome-specific DNA probes and enzyme-mediated (e.g., peroxidase) immunohistochemical procedures.[44~46] This technique now allow direct visulization of chromosome changes in normal, premalignant and tumor tissue without loss of tissue architecture.

In-situ hybridization with chromosome-specific, centromeric DNA probes was used to determine whether chromosome copy number changes could be detected in histologically normal epithelium and cervical intraepithelial neoplasia adjacent to carcinoma of cervix. A important finding of this study is that the amount of genetic change increased as the tissue progressed from normal to CIN to carcinoma. This finding supports the notion of multistep tumorigenesis in cervical region. To present, studies do suggest, however, that the degree of genetic instability increases with histologic stage. The observation of chromosome number abnormalities in the normal and CIN lesions adjacent to the tumor supports the notion of field cancerization. Since this epithelial field harbors genetic alterations, the entire exposed region is an increased risk for developing multiple independent foci of lesions initiated toward malignancy. The finding reported here that CIN tissue harbors nearly the some degree of chromosome polysomy as does the carcinoma region suggests that many of the karyotypic complexities may have occured prior to carcinoma development. While genetic damage may occur throughout the carcinogen-exposed tissue, only cells that have functionally critical genetic lesions may clonally evolve and eventually develop into a tumor.[47]

The study reported here offers major important findings. Genetic alterations,visualized as increases in chromosome 9 copy numbers, were observed in the histologically normal appearing epithelium adjacent to the tumor in all of cases. This measured incidence of polysomy in the histologically normal appearing epithelium adjacent to the tumor can really be considered an underestimate of the true polysomy incidence because the data were obtained on tissue sections containing truncated nuclei and probes for only one chromosome were utilized in this study. One of the goals of this study was to identify genetic biomarkers that might be useful for assessing the risk of tumor development in histologically normal adjacent tumor tissue. The findings of chromosome polysomies in histologically normal epithelium adjacent carcinoma tissue and an increased degree of chromosome polysomy as tissue progressed toward malignancy suggest that the measurement of generalized chromosomal polysomy by in situ hybridization might provide such a genetic biomarker. The advantage of such a biomarker is that it permits the sensitive detection of infrequent events (reflecting accumulated genetic damaged or genomic instability). Individuals whose normal or

CIN epithelium exhibits the greatest degree of genetic abnormalities might be expected to be at the highest risk for tumor development. General genomic instability may be the most important biological marker of all, since it may serve as a marker of the sum of changes in all other categories.

Similar studies in head and neck cancer showing chromosome polysomy in neck cancer specimens have been published.[48] For cervical cancer, chromosome polysomy has been analyzed only in cytology specimens.[49] No published studies have analyzed the chromosome polysomy in cervical biopsy specimens.

Moreover, the frequency of cells with polysomy increased as the tissues passed from histologically normal epithelium to CIN to cancer. The finding of genotypic abnormalities in histologically normal and CIN adjacent to the tumor supports the concept of field cancerization. The finding of progressive genetic changes as the tumor develops supports the concept of multistep carcinogenesis in cervix. Those cases which have invasion had higher mean-polysomy indices and mean-normalized chromosome index that those cases which did not have invasion. Premalignant lesions with higher polysomy index and normalized chromosome index are more likely to develop cancer.

The study reported here suggests the possibility that the determination of the degree of accumulated genetic abnormalities in cervical tissue might prove to be useful for identifying individuals with the highest risk for development of tumor in the cervix. Such genotypic parameter could serve as a biomarker in the assessment of the risk of progression to malignancy as intermediate end-point in chemoprevention trials.

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