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Chemoprevention Trials and Surrogate End Point Biomarkers in the Cervix

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Cervical cancer is the second most common malignancy in women worldwide and remains a significant health problem for women, especially minority and underserved women. Despite an understanding of the epidemiologic risks, the screening Papanicolaou smear, and morbid and costly treatment, overall survival remains 40%. New strategies, based on the clinical and molecular aspects of cervical carcinogenesis, are desperately needed.

Chemoprevention refers to the use of chemical agents to prevent or delay the development of cancer in healthy populations. Chemoprevention studies have several unique features that distinguish them from classic chemotherapeutic trials; these features touch on several disciplines and weave knowledge of the biology of carcinogenesis into the trial design. In the design of chemoprevention trials, four factors are important: high risk cohorts must be identified; suitable medications must be selected; study designs should include Phases I, II, and III; and studies should include the use of surrogate end point biomarkers. Surrogate end point biomarkers are sought because the cancer develops over a long period of time, and studies of chemopreventives would require a huge number of subjects followed for many years. Surrogate end point biomarkers serve as alternative end points for examination of the efficacy of chemopreventives in tissue.

Key words: chemoprevention, cervical intraepithelial neoplasia, squamous intraepithelial lesion, chemoprevention trials, surrogate end point biomarkers, proliferation markers, regulation markers, differentiation markers, genomic instability markers, fluorescence spectroscopy.

Despite the availability of the screening Papanicolaou (Pap) smear, cervical cancer remains a prevalent health problem among women. It is the second most common malignancy diagnosed among women worldwide; 437,300 women were diagnosed with cervical cancer worldwide in 1985. After declining for several decades,
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Incidence rates are currently increasing in American women.2-5 The SEER program estimates that in 1995, 15,800 women in the United States will be diagnosed with invasive cervical cancer, an increase from 15,000 in 1994 and 13,500 in 1993. Similarly, 4800 women will die of invasive cervical cancer, an increase from 4600 in 1994 and 4400 in 1993. Similar trends are noted for the preinvasive counterpart: 65,000 women will be diagnosed with carcinoma in situ (CIS), an increase from 55,000 in 1994 and 50,000 in 1991.2-5 The reasons for this sudden increase are unknown and must be viewed cautiously; some increase may be due to increases in human papillomavirus infection (HPV) and others to increases in human immunodeficiency virus (HIV).6 An estimated 2.5 million American women will have abnormal Pap smear results in 1995, of which half will have some degree of dysplastic lesion.7

The most important risk factor for cervical cancer is HPV. This association has been consistent independent of HPV assay method and epidemiologic study design.8-10 The most common types of HPV are those classified as high risk (HPV 16, 18, 45, and 56), intermediate risk (HPV 31, 33, 35, 51, 52, and 58), and low risk (HPV 6, 11).10 The high and intermediate risk types have been identified in 77% of high grade cervical intraepithelial neoplasia (CIN) and squamous intraepithelial lesions (SIL) and in 84% of invasive lesions.10 Cohort studies demonstrate that women with HPV infection have 11-60 times increased risk of developing high grade CIN and 15-50 times increased risk of developing invasive cancer than do women without HPV infection.11-14

Despite the fact that the cervix is accessible, that a relatively good screening test exists and has been in use for decades, and that new therapeutic initiatives using surgery, radiotherapy, and chemotherapy have been tried, survival remains a dismal 40% worldwide.1 Novel strategies that require an intimate knowledge of the clinical and molecular basis for cervical carcinogenesis are desperately needed.

Definition of Chemoprevention and Chemoprevention Trials

Chemoprevention refers to the use of chemical agents (micronutrients, pharmaceuticals) to prevent or delay the development of cancer in healthy populations.15 These agents, which block the initiating and promoting events of carcinogenesis, augment the preventive strategy, which includes the avoidance of carcinogens in the environment (referred to as primary prevention) and participation in screening programs (referred to as secondary prevention), hence serving as a tertiary preventive measure.16 Sporn cautioned that intervention in the preneoplastic phase is necessary, stating that "the disease process is carcinogenesis, not invasive or symptomatic cancer. Invasive and metastatic cancer are clinical and pathological end stages, at which it may be too late to prevent further progression."17

Chemoprevention trials have several unique features that distinguish them from therapeutic trials. These features call for the involvement of several disciplines to create trials that include the biology of carcinogenesis in the study design.18,19 A thorough understanding of epidemiology, study design, statistical analysis, clinical medicine, and molecular biology is necessary to optimize what can be learned from these studies.20-25

Chemoprevention studies involve four elements: (1) high risk cohorts; (2) suitable medications; (3) Phase I, II, and III trials; and (4) the use of surrogate end point biomarkers. Theoretically, three groups of patients are eligible for such trials: those who are at high risk for cancer but without a precancerous lesion (Phase III), those with a precancerous lesion (Phases I, II, III), and those with a previous malignancy who are at high risk for a second primary or for recurrence (Phases I, II, III). Risk profiles may be based on genetic factors, lifestyle and environmental exposures, a history of a precursor lesion, or some combination of these.18-24

In contrast to Phase I chemotherapy trials, chemoprevention trials are often dose de-escalating, seeking the lowest dose at which biologic modulation of the marker takes place and tolerating little toxicity. Phase II chemoprevention trials, like Phase II chemotherapy trials, evaluate the effectiveness of drugs in a given organ. In contrast to Phase II chemotherapy trials, however, chemoprevention trials necessitate a concurrent placebo-blinded control group (because of the spontaneous regression sometimes observed for preneoplastic lesions) and the use of surrogate end point biomarkers. Phase III chemotherapy and chemoprevention trials both evaluate the cost-benefit ratio of treatments in multicenter settings. In contrast to Phase III chemother-apy studies, however, which compare agents to standard therapies, chemoprevention studies evaluate cancer incidence reduction and choose groups at high risk for the development of cancer rather than those with preneoplastic lesions. Surrogate end point biomarkers allow trials to be of shorter duration, to require fewer subjects, to be lower in cost, to use small tissue samples, and to aid in learning more about the carcinogenic process.18

Chemoprevention Trials: Cohort Selection

High risk cohorts for trials in the cervix include two groups: patients with high grade lesions and women infected with high risk oncogenic HPV types. The ratio-
nale for using patients with high grade lesions is that such lesions are more likely to progress to invasive cancer; up to 36% of (CIS) lesions progressed in a series of studies in which 353 patients were followed without treatment over periods of 3 to 30 years. Patients with high grade lesions are suitable for Phase I, II, and III trials of chemopreventive micronutrients and medications. The rationale for selecting patients with oncogenic HPV lesions comes from the cross-sectional and cohort studies demonstrating greatly increased risk of CIN and of cervical cancer in women who are HPV-positive compared with those who are HPV-negative.

The cervix has long been thought by pathologists as a model for the progression from mildly dysplastic lesions to severely dysplastic lesions to invasive cancer. The ability of clinicians to observe cervical lesions over time with colposcopy and Pap smears makes the cervix a unique organ that is well suited to the development of chemoprevention trials. Pathobiologic studies of cervical carcinogenesis will surely contribute to our understanding of the neoplastic process and hence speed the development of new preventive and therapeutic strategies.

Chemoprevention may be a treatment of choice in the woman who smokes and has HPV and multifocal intraepithelial neoplasia of the cervix, vagina, and vulva. Many of these patients smoke, up to 44% of patients in our population. Many patients have preneoplastic and neoplastic lesions of the aerodigestive tract. Infection with HPV affects the entire squamous epithelium of the female genital tract, and up to 40% of patients with CIN have lesions of the vagina, vulva, and perianal area.

Chemoprevention Trials: Rationale for Medications

In the last few decades, interest in the relationship between diet and cancer in humans has been very strong. Investigators were initially intrigued by international studies in which large differences among countries in cancer incidence rates were found and by the fact that nutritional correlates exist for many cancers. Nutrients are assumed to affect the carcinogenic process at the cellular level, thus supplementation of nutrients would be expected to prevent or reverse the process of carcinogenesis in the earliest phases. Nutritional studies looking for deficiencies in case patients with preinvasive lesions or cancer compared to control subjects have identified micronutrients of interest for use in chemopreventive studies. Some of the other medications of interest are chemically related to micronutrients: retinoids and vitamin A. Other medications of interest, although not related to micronutrients, are believed to interrupt the carcinogenic process in the earliest phases.

Nutrition Studies Suggest Micronutrients of Interest for Chemoprevention Studies

Nutritional study methodology and nutritional measures have been the subject of many reviews. It is important to understand both nutritional study methodology and nutritional measures to understand the inconsistencies among study results. The most commonly used nutritional measures are the diet record, the 24-hour recall, the food frequency questionnaire, and serum levels of nutrients. The diet record, the 24-hour recall, and the food frequency questionnaire are approximations of the diet over a period of time and thus represent the usual exposure over a longer duration of time. Serum nutrient levels provide an objective, repeatable, biologic measure of a nutrient, but depending on the nutrient of interest, may reflect an exposure of shorter duration.

For a diet record, participants are asked to weigh and record the amounts of food eaten, often for a 3- to 7-day period. The 24-hour recall is an interview about the previous 24 hours in which the participants are asked to recall what and how much they have eaten. Both these measures are often recorded several times throughout the year to include foods eaten in all seasons. The food frequency questionnaire is either an interview or a self-administered tool designed to record a persons usual diet. The items of interest for the study are listed and the participants are asked to record how frequently they consume these foods. On some surveys, the portion size is suggested, and on others an average portion size is used for the calculation of nutrients. In general, the diet record is considered the gold standard. Compared with it, the 24-hour recall tends to underestimate the nutrients of interest, whereas the food frequency questionnaire and diet history tend to overestimate them. The food frequency questionnaire is the most suitable instrument for large-scale epidemiologic studies because it can be self-administered and is designed to reflect average intake. Serum nutrient levels reflect a biologic measure of the nutrient of interest. Values for nutrients in these studies are often made in quartiles; the highest quartile will be compared with the other three quartiles to generate a risk ratio. Surprisingly often, the diet record, 24-hour recall, and food frequency questionnaires correlate poorly with serum nutrient levels. Cohen's Kappa, a measurement of interrater agreement, estimates the agreement between serum nutrient levels and each of three questionnaire
methods in the range of 0.2–0.4, putting the agreement in the low to moderate range.31–35

Many investigators have studied the relationship between nutrition and CIN and cervical cancer. Table 1 summarizes three study designs used in the study of this relationship: the food record, the food frequency questionnaire, and serum nutrient levels.19,24–29 Potischman reviewed the studies using these three designs, acknowledging the inconsistencies in results, and concluded that preformed vitamin A levels do not appear related to risk, whereas vitamin C is associated with decreased risk, particularly among smokers.63 Reduced risk is also noted with various carotenoids and vitamin E. Erythrocyte folate measurements demonstrate a protective effect of folate.61 Within each study design, however, results were inconsistent, with some investigators finding deficiencies and others not. Potischman pointed out that further work needs to be done.

Many chemoprevention trials have been designed to supplement deficiencies of these nutrients. It has been and will be important to measure nutritional intake in patients enrolled in chemoprevention trials of nutritional supplements. For example, if trials using nutritional supplements do not show significant regression of lesions, it could be due to the fact that patients likely to participate in chemoprevention trials are not as nutritionally depleted as those who participate in nutrition studies that typically require fewer visits than do chemoprevention studies. The patient sufficiently motivated to participate in chemoprevention trials may be more aware of her diet.

**Micronutrients and Medications of Interest for Chemoprevention Studies**

Micronutrients and medications are assumed to affect the carcinogenic process at the cellular level. Few studies have focused on the cellular effects of nutritional supplementation on the process of carcinogenesis. In contrast, a few studies have looked at the effect of retinoids.63–66 These types of studies, those that examine the reversal of carcinogenesis in the earliest phases, are important to establish the molecular action of chemopreventives. In the cervix, it will be important to examine how these medications affect HPV at the molecular level.

Beta-carotene is the most active and common carotenoid found in the diet and is a remarkably potent source of vitamin A. It is metabolized to retinaldehyde and then converted to retinol. It is thought to be a promising agent based on data from nutritional studies demonstrating beta-carotene deficiencies in CIN patients compared with control subjects. Of all the nutrients studied, the most consistent relationship across the study designs was that between beta-carotene deficiency and CIN and cervical cancer (Table 1).37,39,40,41,45,47,49–52 However, a recent study of beta-carotene for prevention of lung cancer was negative.67

The retinoid group includes vitamin A and its natural and synthetic analogues. One of these analogues is N-(4-hydroxyphenyl) retinamide (4-HPR). Vitamin A is necessary for the normal growth and differentiation of epithelial tissues. Most of the cellular and molecular mechanisms by which retinoids act are mediated by nuclear retinoic acid receptors. However, 4-HPR may act by means of distinct mechanism from that of retinoic acid. Support for this contention has come from the studies of Delia,62 who has shown that 4-HPR can induce apoptosis in retinoic acid-resistant cells. The substitution of an N-substituted carboxamide group for the terminal carboxyl group is believed to account for the decreased toxicity seen with 4-HPR compared with that of other retinoids, making this drug a good choice for long-term use in chemoprevention studies. Nutritional data showing that vitamin A deficiency increases the relative risk of squamous preneoplastic and neoplastic lesions support their use as chemopreventive agents. Retinoids have also been demonstrated to be effective chemoprevention agents in other organ sites.63 In addition, retinoids may have favorable effects on the growth of HPV, making them of particular interest in the cervix. There are several mechanisms by which retinoic acid may affect the HPV E6 and E7 transforming proteins. Bartsch et al.64 demonstrated decreased expression of HPV messenger RNA in the presence of retinoic acid. Retinoic acid has also been shown to increase the secretion of transforming growth factor–beta in cells immortalized by HPV. Transforming growth factor–beta can suppress the expression of the E6 and E7 proteins in cervical epithelial cells.65,66 Thus, the measurement of these factors might serve as markers of responsiveness.

Folate, specifically erythrocytic folate, similar to beta-carotene, has been shown to be deficient in CIN patients compared with control subjects, and these data have supported folate supplementation as a chemopreventive agent.55 Erythrocytic folate levels below 660 nmol/l were shown to enhance the susceptibility of patients to HPV, because folic acid acts as a coenzyme in DNA synthesis for normal cellular growth, proliferation, and differentiation. Pietrantoni et al. studied the regulation of HPV oncogene expression by folic acid. They studied c-fos, c-jun, and HPV E6 expression in Caski (HPV 16-positive) cell lines treated with folic acid. They found diminished c-fos and c-jun expression using Western blot when concentrations of folate of more than 100 nM were used. Similarly, E6 protein expression was diminished at concentrations of more than
Table 1. Studies of Nutrition, Cervical Intraepithelial Lesion, and Cervical Cancer

<table>
<thead>
<tr>
<th>Author</th>
<th>Study design (case controls)</th>
<th>Subjects/controls</th>
<th>Disease</th>
<th>Nutritional element measured</th>
<th>Statistically significant association</th>
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<td>β-Carotene</td>
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Table 1 (continued). Studies of Nutrition, Cervical Intraepithelial Lesion, and Cervical Cancer

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<td>β-Carotene</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>α-Tocopherol</td>
<td>+</td>
</tr>
<tr>
<td>Palan</td>
<td>Cross-sectional, hospital based</td>
<td>80/36</td>
<td>CIN, ICC</td>
<td>Total carotenoids</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>β-Carotene</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>β-Carotene</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cryptoxanthin</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lycopene</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Retinol</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lutein</td>
<td>-</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>α-Tocopherol</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>β-Tocopherol</td>
<td>+</td>
</tr>
<tr>
<td>Batieha</td>
<td>Case-control, population based</td>
<td>50/100</td>
<td>CIN, ICC</td>
<td>Selenium</td>
<td>-</td>
</tr>
<tr>
<td>Potischman</td>
<td>Case-control, hospital based</td>
<td>696/1217</td>
<td>ICC</td>
<td>α-Carotene</td>
<td>-</td>
</tr>
<tr>
<td>Van Eewyk</td>
<td>Case-control, hospital based</td>
<td>(98/98 vitamin C (68/68 folate))</td>
<td>CIN</td>
<td>Total carotenoids</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>β-Carotene</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>β-Carotene</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cryptoxanthin</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lycopene</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Retinol</td>
<td>+</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Lutein</td>
<td>+</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>α-Tocopherol</td>
<td>-</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>γ-Tocopherol</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vitamin C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Folate</td>
<td>+</td>
</tr>
</tbody>
</table>

+: present; -: absent; CIN: cervical intraepithelial lesion; ICC: invasive cervical cancer; CIS: carcinoma in situ.

100 nM, suggesting that the mechanism by which the transcription regulators c-fos and c-jun were controlled was diminished by viral E6 expression.68

Alpha-difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase (ODC). Ornithine decarboxylase is a key enzyme in the biosynthesis of polyamines (putrescine, spermidine, and spermine) and is considered a putative proto-oncogene.
crucial for the regulation of cell growth and transformation.\(^6\) Blocking endogenous ODC prevented transformation of rat fibroblasts by the temperature-sensitive v-src oncogene. The goals of using DFMO to block polyamine-directed transformation are (1) to inhibit transformation under the influence of field cancerization, and (2) to remove cells already transformed by apoptosis.\(^7\) Tumor formation in experimental animals is prevented by inhibitors of ODC such as DFMO,\(^71,72\) and DFMO has been shown to decrease growth of cervical cancer cell lines, regardless of HPV positivity. (Hamada K. Unpublished data.)

The nonsteroidal anti-inflammatory drugs are chemopreventive and are thought to play a role in the control of neoplastic and nonneoplastic cell proliferation and immune function through the inhibition of endogenous prostaglandin biosynthesis. They are extremely well tolerated medications and must be tested as chemopreventives in the cervix.

**Chemoprevention Trials in the Cervix (Phases I, II, III): Past, Present, And Future**

In studies of Phase I, II, and III trials of the cervix (Table 2), patients with CIN lesions were chosen as the high risk cohort. Promising chemopreventive agents that have been or are being investigated in the cervix are retinoids (retinyl acetate gel, all-trans-retinoic acid, 4-HPR), micronutrients (beta-carotene, folate, vitamins), and polyamine synthesis inhibitors (DFMO).\(^22\) These studies have concentrated on histologic and colposcopic regression as the end points. None of these studies have used surrogate end point biomarkers or HPV typing, because these were evolving concepts during the duration of these studies.

**Retinoid Chemoprevention Studies**

Romney et al.\(^80\) reported on a Phase I–II trial using retinyl acetate gel topically in patients with CIN 1–2. Patients treated themselves for 7 days for three sequential menstrual cycles by placing the gel intravaginally. Doses included placebo and 3, 6, 9 and 18 mg of retinyl acetate gel per 6 g of inert vehicle. No serious side effects were noted; approximately half the participants noted vulvar irritation and itching at the 18-mg dose. Only 14% of patients had vaginal burning at any dose during the trial. The study showed that high compliance could be achieved and determined the dose of 6 mg for a Phase III trial.\(^23\) There is no published report of the Phase III trial.

Phase I and II trials by Meyskens et al., Surwit et al., and Weiner et al. demonstrated that all-trans-retinoic acid could be delivered topically to the cervix safely in a cervical sponge and cervical cap. Patients with CIN 1, 2, and 3 were treated by the investigators for 4 days with increasing dosages of topical all-trans-retinoic acid ranging from 0.05–0.484%. They were then seen at 1 week and 1 month for follow-up. Roughly one third of patients experienced vaginal irritation, and roughly half had vaginal burning; only one patient was asked to discontinue the treatment because of these symptoms. A regression rate of 45% was noted in patients treated with doses of 0.15–0.48% compared with a regression rate of 14% in doses lower than these. The optimal dose for a Phase III study was determined to be 0.372%,\(^74,76\)

Meyskens recently reported the results of the randomized Phase III trial of topical 0.375% all-trans-retinoic acid in 141 patients with CIN 2 lesions and 160 patients with CIN 3 lesions. Patients with CIS were excluded from the study. Patients were initially treated with 0.375% all-trans-retinoic acid daily for 4 days, then treated for 2 days each at 3- and 6-month follow-up visits. Patients were seen for Pap smear and colposcopy at 9, 12, 15, 21, and 27 months. Biopsies were performed at the 15-month visit. Many patients were lost to follow-up. Of 151 patients randomized to receive placebo, 81 were evaluated at 15 months and 25 at 27 months. Of 150 patients randomized to receive all-trans-retinoic acid topically, 88 were seen at 15 months and 21 at 27 months. There was a statistically significant regression in the CIN 2 lesions but not in the CIN 3 lesions.\(^77\) Sporn speculated in an editorial that the reason CIN 2 responded and CIN 3 did not was that lesions farther along toward neoplasia may be harder to regress, requiring higher doses, longer administration, systemic administration, or two agents instead of one.\(^74\)

A Phase II study of 4-HPR (supplied by the NCI) is about to commence at the University of Texas M.D. Anderson Cancer Center (Houston). The medication is given orally and thus the effects are systemic. In this study, patients will undergo a complete medical history, nutritional survey, sexual behavior interview, physical exam, colposcopy, colposcopically directed biopsies, HPV testing, blood counts, serum chemistries, nystagmop testing, and smoking cessation counseling. In the Phase II study, 4-HPR or placebo will be given for 6 months. Patients will be followed at 3-month visits for 1 year with the aforementioned tests. Crossover will take place for progression by cytology, colposcopy, or biopsy. Other surrogate end point biomarkers under study in our trial include quantitative cytology and histology (nuclear texture, size, and density); biologic measures of proliferation (proliferating cellular nuclear antigen [PCNA]), regulation (epidermal growth factor receptor, retinoic acid receptor), differentiation (involutrin, cornifin), and genetic instability (chromosome pol-
Table 2. Chemoprevention Trials in the Cervix: Past, Present, and Future

<table>
<thead>
<tr>
<th>Author</th>
<th>Study design</th>
<th>No. of patients</th>
<th>Medication</th>
<th>Disease</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romney$^{96}$</td>
<td>Phase I–II</td>
<td>50</td>
<td>Retinyl acetate gel topically</td>
<td>CIN 1–2</td>
<td>Selected 9 mg dose</td>
</tr>
<tr>
<td>Surwit$^{91}$</td>
<td>Phase I</td>
<td>18</td>
<td>All—TRA topically</td>
<td>CIN 2–3</td>
<td>11% Complete response</td>
</tr>
<tr>
<td>Meyskens$^{82}$</td>
<td>Phase I</td>
<td>35</td>
<td>All—TRA topically</td>
<td>CIN 1–2</td>
<td>Selected 0.372% dose</td>
</tr>
<tr>
<td>Weiner$^{83}$</td>
<td>Phase I</td>
<td>36</td>
<td>All—TRA topically</td>
<td>CIN 1–3</td>
<td>0.05–0.12%</td>
</tr>
<tr>
<td>Meyskens$^{84}$</td>
<td>Phase III</td>
<td>141 CIN 2</td>
<td>All—TRA topically</td>
<td>CIN 2–3</td>
<td>0.15%–48%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>160 CIN 3</td>
<td></td>
<td></td>
<td>48%</td>
</tr>
<tr>
<td>Butterworth$^{96}$</td>
<td>Phase II</td>
<td>147</td>
<td>Folate, 10 mg</td>
<td>CIN 1–2</td>
<td>Folate 14%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vitamin C, 10 mg</td>
<td></td>
<td>Placebo 41%</td>
</tr>
<tr>
<td>Butterworth$^{83}$</td>
<td>Phase II</td>
<td>177</td>
<td>Folate, 10 mg</td>
<td>CIN 1–2</td>
<td>Folate 64%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vitamin C, 10 mg</td>
<td></td>
<td>Placebo 66%</td>
</tr>
<tr>
<td>Childers$^{86}$</td>
<td>Phase III</td>
<td>331</td>
<td>Folate, 5 mg</td>
<td>HPV, CIN 1–2</td>
<td>Folate 7%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Placebo</td>
<td></td>
<td>Placebo 6%</td>
</tr>
<tr>
<td>Romney$^{90}$</td>
<td>Phase I–II</td>
<td>138 proposed</td>
<td>β-carotene</td>
<td>CIN 2</td>
<td>NA</td>
</tr>
<tr>
<td>Berman$^{81}$</td>
<td>Phase II</td>
<td>60 proposed</td>
<td>β-carotene</td>
<td>CIN 2–3</td>
<td>NA</td>
</tr>
<tr>
<td>SWOG$^{82}$</td>
<td>Phase III</td>
<td>225 proposed</td>
<td>β-carotene</td>
<td>CIN 1–2</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vitamin C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Both</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Placebo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitchell</td>
<td>Phase I</td>
<td>30–60 proposed</td>
<td>Efornithine</td>
<td>CIN 3</td>
<td>NA</td>
</tr>
<tr>
<td>Mitchell</td>
<td>Phase II</td>
<td>60 proposed</td>
<td>Efornithine</td>
<td>CIN 3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Placebo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitchell</td>
<td>Phase II</td>
<td>60 proposed</td>
<td>4–HPR</td>
<td>CIN 3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Placebo</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CIN: cervical intraepithelial neoplasia; TRA: transretinoic acid; HPV: human papillomavirus; NA: not available.

ysomy, aneuploidy); and fluorescence spectroscopic emission.

**Micronutrient Chemoprevention Studies**

Romney et al. are conducting a Phase II trial of beta-carotene at 30 mg/day in patients with CIN 2, and 138 patients are expected to be accrued.79 Manetta and Berman have undertaken a Phase II study of beta-carotene in patients with CIN 2–3; 60 patients are expected to be accrued.80 The Southwest Oncology Group has a Phase III study of oral beta-carotene, vitamin C, the combination of beta-carotene and vitamin C, or placebo in patients. The final study design is being revised.81 Folate supplementation was thought to be a good choice for chemopreventive studies showing decreased erythrocytic folate levels in women with CIN.81 Butterworth published an update of a Phase II randomized trial in which patients with CIN 1 and 2 lesions were treated with folate (10 mg), whereas vitamin C (10 mg) was given as placebo, each for 90 days.82 An initial report on the study revealed that folate-supplemented patients were likely to experience cytologic regression of lesions.83 In the final report, there were no statistically significant differences in regression of lesions in the 177 evaluable patients treated with folate or vitamin C.82 A second Phase III multicenter study of folate supplementation by Childers and Chu has had similarly negative results. In this intergroup Southwest Oncology Group study, 331 patients with koilocytic atypia, CIN 1, and CIN 2 were randomized to 5-mg folic acid and placebo. Regression was of borderline statistical significance (P = 0.08) at the 3-month visit, and no difference between groups was noted at the 6-month visit.84

**Polyamine Amine Synthesis Inhibitor Chemoprevention Studies**

Studies underway at our institution include Phase I and II studies of DFMO. The medication is given orally and thus the effects are systemic. In the Phase I study, DFMO is being administered to patients at five dose lev-
surrogate end point biomarkers are deemed useful in chemoprevention trials, however, several questions must be answered. The surrogate end point biomarker must be differentially expressed in normal and high risk tissue; should appear at a well defined stage of carcinogenesis; must provide acceptable sensitivity, specificity, and accuracy; must be easily measured; should be modulated by chemopreventive agents; and should correlate with a decrease in cancer incidence rate. Several types of surrogate end point biomarkers are already in use in clinical trials. A complete list of potential surrogate end point biomarkers for the cervix are discussed in Table 3.

Because HPV is critically important in the carcinogenesis of cervical cancer, surrogate end point biomarkers must be studied in relationship to HPV. zur Hausen and de Villiers have written an important review on the subject. Specific viral transforming genes E6 and E7 from HPV types 16 and 18 act as oncogenes; their expression emerges as necessary, but not sufficient, for malignant conversion. There is no consistent locus in the host for viral integration; however, there is a striking pattern of integration with respect to the viral genome. Integration frequently disrupts the E1 and E2 open reading frames; disrupting these regulatory genes and their regulatory proteins annuls regulation of gene expression. E6 shares functional and structural features of SV40 large T antigen and adenovirus 5 E1B and in vitro promotes degradation of p53 through the ubiquitin-dependent protease system. E7 shares functional and structural features of adenovirus E1A and can complex with retinoblastoma protein. E6 and E7 have been found to stimulate cell proliferation and are responsible for the genetic instability of the infected cell. The transforming gene’s transcriptional and functional activity is regulated by the host cell genome. Mutational modifications of the host cell genome appear to be required for progression to invasion.

Table 3. Classes of Biomarkers in Cervical Epithelium

<table>
<thead>
<tr>
<th>Classes of Biomarkers in Cervical Epithelium</th>
<th>Quantitative Histopathologic and Cytologic Markers</th>
<th>Prognostic Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei—Abnormal size, shape, texture, pleomorphism</td>
<td>Tumor suppressors (p53, Rb)</td>
<td>PCNA</td>
</tr>
<tr>
<td>Nucleoli—Abnormal number, size, shape, position, pleomorphism</td>
<td>Oncogenes (ras, myc, c-erb-2, src, jun, fos)</td>
<td>Ki-67</td>
</tr>
<tr>
<td>Proliferation Markers</td>
<td>Altered growth factors and receptors (EGFR, RAR)</td>
<td>Thymidine labeling index (thymidine, BrdU)</td>
</tr>
<tr>
<td></td>
<td>Polyamines (ODC, arginine, ornithine, putrescine, spermine, spermidine, N1 acetylspermidine)</td>
<td>Mitotic frequency (MPM-2)</td>
</tr>
<tr>
<td></td>
<td>Arachadonic acid</td>
<td>Regulation Markers</td>
</tr>
<tr>
<td></td>
<td>Differentiation Markers</td>
<td>Tumor suppressors (p53, Rb)</td>
</tr>
<tr>
<td></td>
<td>Fibriarl proteins (cytokeartins, involucrin, corninfil, filaggrin, actin microfilaments, microtubules)</td>
<td>Oncogenes (ras, myc, c-erb-2, src, jun, fos)</td>
</tr>
<tr>
<td></td>
<td>Adhesion molecules (cell–cell: gap junctions, desmosomes) (cell–substrate: integrins, cadherins, laminins, fibronectin, proteoglycans, collagen)</td>
<td>Altered growth factors and receptors (EGFR, RAR)</td>
</tr>
<tr>
<td></td>
<td>Glycoconjugates (lectins, mucins, blood group substances, glycolipids)</td>
<td>Polyamines (ODC, arginine, ornithine, putrescine, spermine, spermidine, N1 acetylspermidine)</td>
</tr>
<tr>
<td></td>
<td>General Genomic Instability Markers</td>
<td>Arachadonic acid</td>
</tr>
<tr>
<td></td>
<td>Chromosome aberrations (AgNORs, micronuclei, three-group metaphases, double minutes, deletions, insertions, translocations, inversions, isochromosomes)</td>
<td>Mitotic frequency (MPM-2)</td>
</tr>
<tr>
<td></td>
<td>DNA Abnormalities (DNA hypomethylation, LOH, point mutations, gene amplification)</td>
<td>Regulation Markers</td>
</tr>
<tr>
<td></td>
<td>Aneuploidy (measured by flow cytometry, image analysis)</td>
<td>Tumor suppressors (p53, Rb)</td>
</tr>
<tr>
<td></td>
<td>Fluorescent Spectroscopic Emission</td>
<td>Oncogenes (ras, myc, c-erb-2, src, jun, fos)</td>
</tr>
</tbody>
</table>

Table 3 continued...

Quantitative Histopathologic and Cytologic Markers

Quantitative histopathologic markers include nuclear grading, shape, area, optical density, texture, nuclear pleomorphism, ploidy, and nucleolar size, shape, and position. One of the motivations for the development of the field of quantitative cytology and pathology was to objectify the art and science of these fields of study. Agreement between pathologists can sometimes be difficult to obtain. For example, in studies of readings of cervical smears, Cohen’s Kappas for agreement average 0.4 (moderate agreement), and the results are similar for readings of cervical biopsies. Several investigators have made important contributions to the field of quantitative pathology, including...
Bacus, Palcic, Bartels, Baak, Becker, Bibbo, and Hanselaar. Some are optical engineers who have created the instruments, designed stains for optimizing tissue differences, analyzed the statistical issues of measurements, and written software programs for diagnostic algorithms. Palcic has taken a particular interest in the diagnosis of the screening Pap smear and has developed a camera and software especially refined for the diagnosis of a texture feature called "malignancy-associated change." Some are pathologists with expertise in all areas, including pathology, instrumentation, statistical analysis, and quality assurance in measurement. This is a complicated area of medicine and engineering that requires a diverse set of skills.

Becker investigated the use of nuclear and cytologic parameters, such as size, density, nuclear/cytoplasmic ratio, and texture features, to make more objective assessments of the histopathologic continuum from preneoplastic to neoplastic lesions. Using computer-assisted image analysis for morphometric measures and regression analysis as a statistical method, he was able to predict survival in 70-80% of uveal melanomas, to correctly classify 97% of benign and malignant endometrial specimens, to predict recurrences in malignant fibrous histiocytoma, and to categorically agree with the pathologist in 95% of sclerosing adenosis and tubular breast carcinoma cases. His studies have helped establish the validity of this approach in the literature.

Barts has written extensively on the optimization of image analysis in histopathology. These articles are of particular interest for the clarity they bring to a field in which diverse areas of expertise are necessary. The issues of statistical analysis are particularly well described and are not to be underestimated, for the complexity involved in the data reduction step alone is staggering. In two studies, Bibbo, Bartels, and their collaborators examined areas adjacent to cervical CIS and invasive cancer and found changes in karyometric marker features in the nuclei of histologically normal-appearing cells. The best marker features by discriminant analysis were nuclear roundness, nuclear perimeter length, total optical density, and a texture measure. This is of particular interest in chemopreventive studies; modulation of these features can be a biomarker of regression.

Hanselaar and colleagues have a long-standing interest in measurements of the cervix. They examined nuclear features and compared them to ploidy analyses in the cervix. They concentrated their efforts on high grade lesions and lesions adjacent to invasive cancer. They found the best predictors of aneuploidy to be nuclear area and circumference, texture, and density.

Other authors, using image analysis systems, have had similar results. Morphometry may be the objective diagnostic technique long needed to objectify the subtle pathologic measures that predict lesions likely to progress: increased nuclear size, increased nuclear density, and abnormal nuclear texture. These changes, when present, distinguish lesions that are more likely to progress to invasion—changes that reflect underlying genetic change affecting growth control.

**Proliferation Markers**

The rationale for the use of proliferation markers is that cells with high proliferative activity are more likely to be associated with premalignant and malignant tissues. Proliferation is thought to be an early marker of disordered growth. It is hypothesized that increased proliferation is associated with more advanced lesions and that the distribution of proliferating cells in tissue may tell us about the regulatory mechanisms that become dysfunctional during the multistep process. Proliferation can be studied with Ki-67 and PCNA in archival specimens. The distribution of proliferation by layer (basal vs. parabasal versus superficial) might indicate growth regulatory mechanisms, thus the relationship of proliferation to growth dysregulation is of interest.

**Proliferating Cellular Nuclear Antigen**

Proliferating cellular nuclear antigen is a nuclear protein whose expression is associated with the late G1 S and early G2 phases of the cell cycle. An auxiliary protein to DNA polymerase, it plays a critical role in initiating cellular proliferation. Studies of PCNA in cervical specimens have recently been published and demonstrated increased activity as the lesions progress to invasion. Ahn et al. have undertaken studies of PCNA in invasive cervical cancer specimens from patients treated with retinoids and interferon and have found that PCNA is highly expressed in these lesions. It appears to be a promising measure of proliferative activity in the cervix; the correlation of proliferation with regulatory and differentiation markers will be important for understanding the neoplastic process.

**Ki-67**

Konishi et al. studied the presence of Ki-67 in samples from normal, dysplastic, and cancerous cervixes. Ki-67 was not quantitatively measured, but increases were noted during the normal menstrual cycle and as lesions progressed to cancer. The work of Konishi et al. demonstrates that studies of proliferation markers need to control for age and the time in the menstrual cycle. Ki-67 was quantitatively measured by image analysis by...
Devictor et al. and was found to be statistically significantly increased as lesions progressed from CIN 1 to microinvasive cancer; no relationship to HPV was noted.138

Labeling Indexes

Tritium has been used as a label for high-resolution autoradiography and, when combined with thymidine, selectively labels nuclei that are synthesizing DNA. It is stable in the nucleus after incorporation and produces relatively little disturbance in the mitotic cycle. Richart conducted a study of CIN lesions in which lesions were chosen ranging from normal to CIS; there were six levels of pathologic severity diagnoses for the study. The lesions were stained with tritiated thymine and measured radioautographically. A linear relationship between log of labeling index and severity of lesion was demonstrated. The calculations of doubling time for normal cervix was 5.7 days, whereas that for CIS was estimated to be 11.3 hours.119

Bromodeoxyuridine is a thymidine analogue that is also incorporated into nuclear DNA. A monoclonal antibody identifying bromodeoxyuridine-containing nuclei was used in an immunohistochemical study of cellular proliferation. Fukuda et al.120 used this antibody to study S-phase labeling in cone biopsy specimens. The levels of bromodeoxyuridine-positive cells were 5.1% in normal epithelium, 12.3% in slight to moderate dysplasia, and 21.2% in severe dysplasia and CIS. In normal tissues, the majority of bromodeoxyuridine-staining cells were confined to the parabasal layer, whereas increases through the intermediate layer extending to full thickness involvement were seen as lesions progressed to CIS.120

Mitotic Frequency

MPM-2, a mitotic monoclonal antibody, recognizes a phosphorylated epitope on a group of proteins that are phosphorylated at mitosis. Because it preferentially decorates cells in mitosis, the relationship of mitosis to histopathologic change can be examined. In a feasibility study conducted on 23 cervical cone biopsies, Hu et al.121 studied mitotic figure frequency per unit of epithelium. They found statistically significant increases in MPM-2 staining as lesions progressed from normal to CIN 1, 2, 3 to invasive cancer; these quantitative measurements were obtained using computer-assisted image analysis. Moreover, the relative distance of the mitotic cells away from the basal layer increased with the severity of the lesion, from CIN 1 to invasive cancer. Mitotic cells in high grade lesions were distributed across the full thickness of the epithelium. These results suggest that proliferation becomes sequentially dysregulated numerically and spatially during cervical carcinogenesis.121

Regulation Markers

Regulation markers include tumor suppressors, oncogenes, growth factors and their receptors, and polyamines. These agents in their normal state help regulate cell growth. Their measurement may provide clues to the process of carcinogenesis. If they are found to be present differentially in high grade lesions compared with low grade lesions, they will be useful as surrogate end point biomarkers.

Tumor Suppressors

The p53 tumor suppressor gene is believed to be responsible for more than 50% of human cancers. Cervical cancer is the exception. Human papillomavirus E6 has been shown to functionally repress the action of wild-type p53.122,123 Studies of cervical cancer show that most cervical tumors (95%) are HPV-positive and do not overexpress p53.130 Similar findings exist for CIN lesions.131,132 Those tumors that are HPV-negative are more likely to have p53 mutations, but not exclusively so.133-136 One can observe p53 mutations in metastatic lesions, thereby suggesting a later role for full p53 alterations.

The retinoblastoma (Rb) gene is a tumor suppressor that is inherited dominantly, although the disease is recessive. The active form of Rb is thought to be a dephosphorylated form of the protein that accumulates in the cell in the G0/G1 phase of the cycle. It has been shown that HPV E7 functionally represses the action of Rb. If regulation of the cell cycle can be negated by HPV E7, then mutations of Rb may not be necessary in cervical cancers. Heck et al. and Scheffner et al. reported no gross Rb-1 abnormalities or 13q allelic loss regardless of HPV status in 28 invasive cervical cancers.137,138

Oncogenes

Ha-ras has been demonstrated to be overexpressed in cervical cancers and high grade lesions compared with that in the normal cervix.139-145 Several laboratories have identified H-ras codon 12 mutations in cervical cancers, and Van Le et al. were unable to find them in CIN lesions, thus suggesting that ras mutations are late events.144

The c-myc oncogene was measured quantitatively and shown to increase as lesions progressed from CIN to microinvasion.118,146-149 Riou and Iwasaka and colleagues demonstrated that c-myc overexpression is as-
sociated with relapse and metastatic potential in invasive cervical cancers.\textsuperscript{146,148}

Hale et al. found \textit{c-erbB-2} expressed in 39\% of invasive cervical cancers. The authors noted a correlation with survival.\textsuperscript{150} Mitra evaluated a panel of 22 protooncogenes in 50 cervical cancers and found amplification of the genes for \textit{c-erbB-2} in 14\%; amplification ranged from 5 to 68 copies, suggesting an important role in carcinogenesis.\textsuperscript{151} In addition, \textit{c-erbB-2} oncogenes were found to be overexpressed in cervical carcinomas, with pronounced overexpression in aneuploid tumors.\textsuperscript{139,152,153}

\textbf{Altered Growth Factors and Receptors}

The \textit{erbB-1} oncogene was discovered as one to two oncogenes carried by the avian erythroblastosis virus, for which the protooncogene was found to encode a membrane-associated tyrosine kinase protein that was identified later as the EGF receptor.\textsuperscript{154-157} The HPV E5 gene has been found, when expressed from a heterologous promoter, to be oncogenic and to cooperate with EGF to further enhance transformation.\textsuperscript{158,159} Levels of EGFR are similarly increased as lesions progress from early dysplasias to invasion.\textsuperscript{160}

Retinoic acid receptors have been found to be important in predicting response to retinoids in aerodigestive tract tumors and have been shown to mediate decrease growth in cervical cancer cell lines treated with retinoids.\textsuperscript{161} Cellular retinoid and retinol-binding proteins (CRABP, CRBP) have been studied in CIN and invasive cancer. Hillemans et al. studied CRABP and CRBP and found the CRABP 1 was present in the basal layer of normal cervix and low grade CIN, whereas the distribution was altered in high grade CIN and cancer, in which it was found to extend to the superficial layer.\textsuperscript{162} Retinoid status has been found to control the appearance of reserve cells and keratin expression in mouse cervical epithelium.\textsuperscript{163} Agarwal et al. proposed that retinoids act by reducing the extent of viral oncogene transcription and thus slow the neoplastic process.\textsuperscript{164}

\textbf{Polyamines}

Polyamines (putrescine, arginine, ornithine, spermidine, and spermine) play a critical role in cellular maintenance, proliferation, differentiation, and transformation. A key enzyme in polyamine biosynthesis, ODC is considered to be a proto-oncogene crucial for regulation of cellular growth and transformation.\textsuperscript{69} Cancer patients have elevated levels of polyamines in their physiologic fluids compared with their normal counterparts. A specific “suicide inhibitor” of ODC, DFMO exhibits antitumor and antimetastasis activities and is effective in many carcinogen-induced animal chemoprevention models.\textsuperscript{71,72} Preliminary studies by Nishioka et al.\textsuperscript{165} indicated that ODC activity and polyamine levels could be measured with the amounts of tissue obtained by routine cervical biopsy (1.3–11.1 mg). However, they then observed the presence of cadaverine, which indicated that bacterial contamination was present; this finding is expected in the cervix but is a problem for polyamine measurement, because some of the polyamines present might be coming from the bacteria. Thus, Nishioka et al. decided to rinse tissues before freezing them. As expected, they observed increases of plasma precursor amino acids of polyamines, such as arginine and ornithine, at even very low doses of DFMO. They were also able to measure DFMO itself as a compliance marker. The tissue measurements have shown wide variability, as expected and as noted in other organ sites in which DFMO studies have been undertaken.\textsuperscript{166} The spermidine/spermine ratio will be evaluated for tissue measurements as a way of decreasing variability.

Nishioka et al. also examined tissue N\texttextsuperscript{1}-acetylspemidine because transformed NIH 3T3 cells excrete N\texttextsuperscript{1}-acetylspemidine\textsuperscript{167} and N\texttextsuperscript{1}-acetylspemidine is primarily found in tumor tissue, not in normal tissue.\textsuperscript{168,169} They noted detectable N\texttextsuperscript{1}-acetylspemidine in some colposcopically normal specimens and most colposcopically abnormal specimens. They were surprised to see that some normal specimens had N\texttextsuperscript{1}-acetylspemidine, but reasoned that the colposcopically normal areas could have been infected with HPV, which can transform cervical cells and thus could be producing N\texttextsuperscript{1}-acetylspemidine. Further tissue studies are underway. On the basis of the results, Nishioka et al. believe that polyamines and their precursor amino acids are effective markers in analyzing the effects of DFMO, functioning as pharmacodynamic parameters as well as biomarkers for transformation in the cervix.\textsuperscript{165}

\textbf{Differentiation Markers}

Differentiation markers include fibrillar proteins (keratins, involucrin, cornifin, filaggrin, actin microfilaments, microtubules), adhesion molecules (cell–cell: lectins, gap junction, desmosomes; cell–substrate: integrins, cadherins, laminins, fibronectin, proteoglycans, collagen), and glycoconjugates (mucins, blood group substances, and glycolipids). If they can be demonstrated to be differentially expressed in lesions more likely to progress to invasion, then they may prove to be useful surrogate end point biomarkers.


Fibrillar Proteins

Keratins are proteins of 40–67 kd organized into filaments that are found in different combinations in human epithelial tissues and whose expression correlates with distinct types of epithelial differentiation. Involutin and cornifin are major protein constituents of human cornified epithelium and undergo cross-linking by epidermal transglutaminase. Antibodies are available to study keratins and involucrin in fixed tissue and transglutaminase in fresh tissue.

Several investigators have looked at keratin expression in cervical epithelium. The cervix appears to have lower molecular weight keratins (39, 43, and 58 kd) than the aerodigestive tract epithelium, for example. The CAM 5.2 antibody binds to these keratins and is expressed differentially in normal, premalignant, and malignant epithelium. The relationship of HPV infection to cytokeratin differentiation is complicated.

Conversely, involucrin, as measured by immunoperoxidase staining by two investigators, is present in almost all normal epithelium and HPV lesions but disappears as lesions progress to CIN and invasive cancer. Keratins, involucrin, and cornifin are of interest as markers, because they are modulated by retinoids. Differential expression of keratins and involucrin has been demonstrated in cervical cancer cell lines. Filaggrin, a differentiation-dependent cytoplasmic protein, is altered in cells with HPV, and its expression decreases as lesions progress to invasive cancer.

Adhesion Molecules

Discrete combinations of adhesion molecules are expressed by endothelial cells at different anatomic sites, and these combinations appear to be responsible for selective recruitment of different leukocyte subpopulations into particular tissues. The upregulation of adhesion molecules by locally released soluble mediators is an important process in enabling numerous leukocytes to congregate at sites of inflammatory or immunologic activity. Adhesion molecules can interact specifically with ligands expressed on the surface of vascular endothelial cells, which include members of the selection family, such as E- and P-selection, and those belonging to the immunoglobulin superfamily, principally ICAM-1, ICAM-2, and VCAM-1. Vascular adhesion molecules are upregulated in high grade CIN lesions but not in low grade lesions.

Integrins are transmembrane glycoprotein heterodimers composed of noncovalently associated a and b subunits, which link the cytoskeleton to the extracellular matrix. The extracellular domains of both subunits contribute to the ligand-binding site, whereas the cytoplasmic domains interact either directly or through linker molecules with the actin cytoskeleton. Three major integrins have been described in epithelial cells: a2b1, a3b1, and a6b4. In squamous epithelium b, integrins are involved in cell–cell contacts, whereas a6b4, a component of hemidesmosomes that may function as a laminin receptor, is involved in the formation of bonds between epithelial cells and basement membranes. De-arrangement in the expression of the tissue-specific integral complement may play a key role in epithelial neoplastic progression, because cell–cell contact affects differentiation and invasive ability. Integrin beta-4 has enhanced expression in high grade lesions and cancers.

Lectins and Glycoconjugates

Lectins and glycoconjugates are aberrantly expressed as neoplastic transformation advances. Pillai et al. studied jack fruit lectin in exfoliated cervical cells of increasing grades of CIN and found no binding in normal cells and intense binding in highly dysplastic cells. Li studied lectin receptors in normal, dysplastic, and neoplastic cervixes with a panel of 12 lectins and demonstrated that some receptors correlated with tumorigenicity, others with differentiation, and others with invasion. Lectins are of interest as markers, because they are modulated by retinoids.

General Genomic Instability

General genomic instability may be the most important biologic marker, because it may serve as a marker of the sum of the changes in all other categories. Aneuploidy is a well established marker of prognosis in other organ sites.

Chromosomal Aberrations

The cell nucleus contains large loops of DNA whose ribosomal rRNA genes are transcribed by RNA polymerase I. Such a loop is known as a nucleolar organizer region (NOR). In humans, NORs are located on the secondary constrictions of acrocentric chromosomes. In the diploid cell, as many as 10 NORs can be observed. The NOR-associated proteins can be stained with silver (AgNORs), which increase as lesions progress from koilocytosis to high grade CIN. Authors differ with respect to the correlation of AgNORs with proliferation.
Micronuclei are intracytoplasmic inclusion bodies formed from chromatin fragments or whole chromosomes. Their presence in cells is a reflection of chromosomal aberrations during cellular mitosis. The micronuclei in exfoliated cells of the cervix have been suggested to represent a marker of malignant potential. CIN 2 and 3 were shown to have higher levels of micronuclei than CIN 1.

Three-group metaphases are morphologically well defined and readily recognizable (by light microscopy) atypical mitotic figures and are associated with aneuploidy. In a study of 72 cone specimens containing CIN, Pieters et al. demonstrated increased three-group metaphases in higher grade CIN, in aneuploid lesions, and in women older than age 35.

**DNA Abnormalities**

Global DNA hypomethylation has been observed in some human neoplasms and has been implicated as an important factor in carcinogenesis. Kim et al. studied whether DNA hypomethylation occurs in CIN and in cancer and determined the relationship between the degree of DNA methylation and the grade of neoplasia. This was measured by \( ^{3} \text{H} \) methyl group incorporation and was found to be increased threefold and sevenfold in the DNA from cervical dysplasias and cancers, respectively, compared with normal cervical tissues.

Studies on loss of heterozygosity (LOH) have revealed losses of genes at specific chromosomes that are commonly altered in human tumors. These changes are thought to be critical for unmasking the recessive genetic changes of carcinogenesis. Yokota et al. demonstrated LOH at the D3S2 locus on chromosome 3p in nine fresh cervical tumors. This locus is also lost commonly in lung cancer and in renal cell carcinoma. Chung et al. similarly found LOH at 3p25 and 3p14. Mitra et al. performed detailed allelotype analysis of DNA from 53 primary cervical cancers and corresponding normal cells and used 57 polymorphic probes mapped to each of the chromosomal arms excluding the short arms of the acrocentric chromosomes. They observed LOH on 10 chromosomal arms, as follows: 1q (26%), 3p (35%), 4q (46%), 5p (53%), 5q (38%), 6p (28%), 10q (28%), 11p (42%), 18p (38%), and Xq (26%). The most frequently occurring LOH in their study was on 4q (ADH3) and 5p (DS519). Although LOH may not itself be a biomarker, it may help us understand the process of carcinogenesis, thus allowing for the discovery of other biomarkers.

**Aneuploidy**

Additional evidence for the histopathologic continuum has been found in DNA ploidy analyses, which have also helped establish the biologic continuum. Some of these studies have been performed with the use of flow cytometry and others with computer-assisted image analysis. In both study methods, a high percentage of CIN lesions have demonstrated aneuploidy. Similarly, both methods have demonstrated aneuploidy in tissue infected with HPV but not with CIN. There remain broad differences in estimates of aneuploidy among studies, however. Three studies looking at ploidy with flow cytometry in CIN 3 lesions reported wide ranges (20–80%) of aneuploidy. Hughes et al. reported that aneuploidy was no more common in CIN 3 (21%) than in HPV-infected cervixes (18%). Willen et al. reported that only 50% of invasive lesions were aneuploid. Watts et al. compared flow cytometric studies with image analysis in specimens divided into two parts and demonstrated that image analysis had greater sensitivity than flow cytometry. They reported that many of the HPV lesions without CIN were aneuploid (47% with flow cytometry, 87% with image analysis). This may show that HPV is truly part of the pathologic continuum to cancer. Hanselaar et al. demonstrated DNA aneuploidy in 89% of samples from women with CIN 3 with or without adjacent invasive cancer. The DNA patterns in the areas of CIN and invasive cancer were identical, suggesting that the lesions were related. It appears that HPV alters the flow cytometric findings sufficiently, thus HPV should always be measured.

Fu et al. conducted a study examining ploidy by image analysis in 100 lesions of CIN, for which there was follow-up information. Diploid and tetraploid lesions regressed 85% of the time, whereas aneuploid lesions persisted or progressed to invasion 85% of the time. Bibbo et al. reported a study similar to that of Fu et al. in which the outcome of lesions was known. There were 211 cases, of which 29% of HPV, 59% of CIN 1, 56% of CIN 2, and 72% of CIN 3 lesions were aneuploid. The patients were clinically followed, and a comparison of 150 abnormal nuclei with 50 normal nuclei was done by image analysis. The aneuploid lesions were found to persist or progress, whereas the polyploid lesions regressed. These studies are extremely valuable in ascertaining the value of these biomarkers in predicting invasion.

Ploidy appears to be a good predictor of biologic behavior and may have better predictive value than histopathologic characteristics judged by the eye. The aforementioned studies of ploidy did not measure HPV consistently, which appears to affect ploidy. These studies need to be validated with larger samples, and researchers must emphasize consistent histopathologic review, control for HPV as measured by polymerase chain reaction, and use image analysis rather than flow...
Cytometric studies because the former is quantitative and reproducible. Genomic instability is a risk factor for invasive cancer, because these abnormalities drive the multistep carcinogenesis process.

**Fluorescent Spectroscopic Emission**

Fluorescence spectroscopy is defined as the use of light to probe the biochemical properties of tissue. Tissue is illuminated with monochromatic light at either ultraviolet or visible wavelengths, and the resulting fluorescence intensity, as a function of wavelength, is measured quantitatively. Qualitative and quantitative differences are present due to changes in tissue scattering (which can reflect changes in cell size, shape, and uniformity), tissue absorption (which can reflect changes in DNA content and hemoglobin), and tissue structure (which can reflect new blood growth, nuclear/cytoplasmic ratio). The fluorescence spectra contain information about the presence of tissue fluorophores (NADH, FADH, elastin, collagen). The penetration of fluorescence at ultraviolet wavelengths of light is limited to several hundred microns and is thus well suited to the detection and diagnosis of intraepithelial lesions.

This technique provides an automated diagnosis in real time with little or no training of the person performing the measurement. Several groups have attempted to use fluorescence spectroscopy to diagnose preneoplasias and neoplasias of the colon, lung, and cervix. Use of fluorescence for the making of diagnoses will allow us to identify lesions before they are visible to the naked eye. Lam et al. have advanced this strategy tremendously in the lung by using image cytometry to identify texture changes in cells in the bronchial epithelium before they are visible to the cytologist and by then using targeted areas for biopsy by means of tissue autofluorescence.

In vitro and in vivo studies of cervical epithelium have demonstrated that measurements at 337, 385, and 460 nm of emission can be used to develop a diagnostic algorithm with comparable sensitivity and superior specificity, positive predictive value, and negative predictive value to that of colposcopy. The value of this technique in chemoprevention studies is twofold: (1) Lesions not visible during examination or during white-light scoping can be targeted for biopsy, and (2) small lesions that might be affected by biopsy can be followed without biopsy.

**Conclusion**

Cervical cancer is the second most common malignancy in women worldwide and may be increasing in the United States due to increases in HPV and HIV infection. Despite a thorough understanding of the epidemiologic risks, decades of use of the screening Pap smear, and new trials of morbidity and costly treatments with surgery, radiotherapy, and chemotherapy, overall survival remains low. New strategies based on the clinical and molecular aspects of cervical carcinogenesis are desperately needed.

Chemoprevention refers to the use of chemical agents to prevent or delay the development of cancer in healthy populations. Chemoprevention studies have several unique features that distinguish them from classic chemotherapeutic trials; these features touch on the disciplines of clinical medicine, molecular biology, epidemiology, and statistics and utilize knowledge of the biology of carcinogenesis in trial design. In the design of chemoprevention trials, four factors are important: high risk cohorts must be identified; suitable medications must be selected; study designs should include Phases I, II, and III; and studies should include the use of surrogate end point biomarkers.

High risk cohorts for cervical chemoprevention studies include women with CIN/SIL. Nutritional studies have helped define micronutrients of interest for these studies: folate, carotenoids, vitamin C, and vitamin E. Medications of interest include retinoids (4-HPR, retinyl acetate gel, topical all-trans-retinoic acid), polyamine synthesis inhibitors, DFMO, and nonsteroidal anti-inflammatory drugs (ibuprofen). Phase I chemoprevention studies on the cervix have tested retinyl acetate gel and all-trans-retinoic acid topically, setting doses for Phase II studies. Phase II trials of topical all-trans-retinoic acid, oral beta-carotene, and oral folic acid continue. Phase III trials of topical all-trans-retinoic acid have been completed and have shown significant regression of CIN 2 but not of CIN 3. Phase I studies of oral DFMO and Phase II studies of oral DFMO and 4-HPR are underway. Oral chemopreventives would be expected to have systemic effects and would thus reverse precancerous lesions in the aerodigestive tract or other HPV-induced lesions of the female genital tract. Surrogate end point biomarkers, intermediate markers of cancer risk, under study include (1) quantitative cytology and histopathology; (2) HPV type testing; (3) biologic measures of proliferation, regulation, differentiation, and genomic instability; and (4) fluorescence spectroscopic emission.

Clinical chemoprevention trials with biologic end points will contribute to our understanding of the multistep neoplastic process, identify precancerous lesions at higher risk for progression to invasion, and provide new targets for intervention, hence contributing to the development of new preventive and therapeutic strategies for the treatment of cervical cancer.
Chemoprevention in the Cervix / Mitchell et al.

Lessons learned from procedures used for the cervix may unravel some of the mystery of squamous carcinogenesis and provide insight into new molecular therapies for other squamous neoplasms.

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