Molecular Epidemiology and Retinoid Chemoprevention of Head and Neck Cancer

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Head and neck cancer is a major worldwide health problem; it has been estimated that approximately 900,000 people were diagnosed with this disease in 1995. Patients are generally treated with surgery and/or radiation therapy. Treatment, especially of patients with early stage (I or II) head and neck squamous cell carcinoma, is often successful. A serious concern, however, is the fact that these patients subsequently develop second primary tumors at an annual rate of 4%-7%. Molecular analyses of premalignant and malignant tissues have produced strong evidence that clonal genetic alterations occur during the early stage of aerodigestive tract carcinogenesis. Although the roles of tobacco and diet in head and neck carcinogenesis have been the subjects of epidemiologic investigations for many years, it has only recently become possible to integrate information regarding genetic susceptibility factors into the development of comprehensive risk models for these cancers. The molecular and epidemiologic studies provide the foundation on which clinical trials can be designed to evaluate the role of retinoids and other compounds in the reversal of premalignancy and the prevention of second primary tumors (i.e., in chemoprevention). This translational approach has led to studies of the utility of intermediate end point markers, such as the nuclear retinoic acid receptors, in chemoprevention strategies. Given the rapid advances occurring in this area of research, it may soon be possible to use these biomarkers to identify patients who are most at risk for developing head and neck cancer and who are most likely to benefit from chemopreventive interventions. [J Natl Cancer Inst 1997;89:199-211]

It has been estimated that in 1995, there were nearly 900,000 new cases of head and neck cancer worldwide, 600,000 in men and 270,000 in women (1). In the United States alone in 1996, head and neck cancers accounted for 3.2% of all incident cancers (39,750 new cases) and 2.3% of cancer fatalities (12,460 deaths) (2). Worldwide, the incidence rates for head and neck cancers have been increasing, as have the mortality rates for patients with these cancers (3). In the United States and other highly developed countries, the current 5-year survival rate of 40% for patients with head and neck cancer is little improved over the 5-year survival rate determined in the 1960s, despite improved methods of detection and local control and the development of new chemotherapeutic regimens with substantial activity in this disease.

Standard care for patients with head and neck squamous cell cancer (HNSCC) is surgery or radiation therapy or a combination of the two (4). This care is frequently successful for the treatment of patients with early stage (I or II) HNSCC (5); disease relapses occur in only 30%-35% of these patients. However, effective standard control and freedom from relapse is no guarantee of long-term survival. Even successfully treated patients with early stage disease face a constant annual 4%-7% risk of developing potentially fatal second primary tumors (SPTs), mostly in foci of smoking-related carcinogenesis, including the aerodigestive tract and bladder.

Standard therapy is far less successful for patients with locally advanced (stage III or IV) HNSCC or metastatic disease (5). Cisplatin-based chemotherapy has been found to decrease the rate of occurrence of distant metastases and to improve organ preservation when combined with radiation therapy (5). Chemotherapy, however, has failed to improve survival for patients with advanced HNSCC. Even when successful, therapy for patients with advanced disease produces substantial functional and cosmetic morbidity that decreases the quality of life. Patients with advanced disease who are successfully treated and who overcome morbid treatment effects that are diminished through use of radiation toxicity-reduction techniques and reconstructive surgery and rehabilitation still face the same specter of SPT risk as patients with early stage disease who have been “cured” (5,6) (Fig. 1).

The primary therapy and relative rate of survival for patients with HNSCC will very likely improve as a consequence of continuing advances in the diagnostic and therapeutic methods available for use in the clinic. A certain consequence of such an improvement will be more patients living long enough to develop SPTs. The compelling data on diagnosis and therapy cited

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See “Notes” following “References.”
Multistep and Field Carcinogenesis

In 1976, Knudson (8) hypothesized that retinoblastoma was a two-hit carcinogenic process. Subsequent work by multiple investigators has revealed that epithelial cancers, including HNSCC, are the end product of a multistep carcinogenic process. The possibility that this process might be blocked, reversed, or inhibited before cells and tissues reach the cancer end stage has been the driving force behind chemoprevention research.

Molecular studies of premalignant tissue, particularly studies of loss of heterozygosity (LOH), provided strong evidence for the concept of multistep carcinogenesis. van der Riet et al. (9) found a high rate of LOH at the human chromosomal locus 9p21 in squamous dysplasia and carcinoma in situ lesions. This LOH rate and location were similar to those in invasive carcinomas. Mao et al. (10) subsequently found LOH at the chromosomal loci 9p21 and 3p14 in 19 (51%) of 37 oral leukoplakia samples examined. Seven (37%) of 19 patients with LOH at one or both of these loci subsequently developed squamous cell carcinoma, whereas only one (6%) of 18 non-LOH patients developed head and neck cancers. These studies suggest that clonal genetic alterations occur at an early, or premalignant, stage of carcinogenesis.

Further evidence that the process of carcinogenesis in head and neck cancers is a multistep process comes from studies of genetic alterations that demonstrate that chromosomal abnormalities (i.e., polysomies) occur not only in tumor cells, but also in histologically defined premalignant lesions, such as oral leukoplakias and nonmalignant epithelial tissue adjacent to a tumor. Hung et al. (11) also found evidence in support of the multistep nature of this process when they demonstrated that highly specific allelic deletions in the short arm of chromosome 3 occur at the earliest stage (i.e., hyperplasia) in the pathogenesis of lung cancers and can be found in cells throughout the respiratory tract.

In addition to multistep carcinogenesis, the concept of field cancerization provides a major rationale for chemoprevention of epithelial cancers. Slaughter et al. (12) proposed this concept in 1953 to explain the development of SPTs associated with oral cancer. Field carcinogenesis is the extensive, multifocal development of premalignant and malignant lesions within the entire carcinogen-exposed area of an epithelial region. The classic example of field carcinogenesis is tobacco-induced injury of the upper aerodigestive tract and lungs.

Blot et al. (13) established the association of mutations of the p53 tumor suppressor gene with cigarette smoking and with HNSCC. They found a high frequency of p53 mutations in patients with HNSCC with 54 (42%) of 129 patients having evidence of a p53 mutation in their tumors. They also found great variability in the site and type of mutation within the gene, which led to studies of p53 mutations to test the concept of field carcinogenesis. According to this model, primary tumors and SPTs are hypothesized to be genetically independent events; therefore, p53 mutations in the primary tumor and SPT(s) of the same patient would be of different types and at different sites.

Chung et al. (14) examined p53 in DNA extracted from the primary tumors and associated SPTs of 31 patients with HNSCC. They reported that primary tumors and associated SPTs had a high (43%) rate of discordance in the site of mutation within the p53 gene. They analyzed the DNA from exons 5-8 of p53 genes from primary tumors and SPTs. Twenty-one of the 31 patients were found to have mutations in the p53 gene. In all 21 patients, discordance in p53 mutation occurred between the primary tumor and associated SPTs, indicating that the development of SPTs is an independent genetic event. The concept of field carcinogenesis was further supported by several subsequent molecular studies, including studies of epithelia distant from the tumor, which also found different p53 mutations in multiple foci. One recent study (15) of the patterns of allelic loss on the short
arms of chromosomes 9 (i.e., 9p) and 3 (i.e., 3p) in multiple primary head and neck tumors, however, suggested that, at least in a proportion of patients, multiple cancers arise from a single clone. The same investigators also evaluated 87 preinvasive lesions and paired normal samples by means of microsatellite analysis to establish the presence of allelic loss at 10 critical genetic loci that are frequently lost in HNSCC cells. Their findings demonstrated that abnormal mucosal cells surrounding preinvasive and microinvasive lesions shared common genetic alterations with those lesions and therefore appeared to arise from a single progenitor clone. On the basis of these findings (15,16), the Johns Hopkins group concluded that the phenomenon of field carcinization appeared to involve the clonal expansion and migration of related neoplastic cells.

The current standard of local and systemic anticancer approaches does not eliminate or ameliorate the major consequence of field carcinogenesis—SPTs. These tumors represent a major cause of failure and death in definitively locally treated primary cases of head and neck cancer. As diagnostic and therapeutic procedures continue to improve, the problem of SPTs will grow, as will the need for chemoprevention and other novel approaches to control them (17).

Epidemiology

Tobacco

The etiologic importance of tobacco use in oral and laryngeal cancers is unquestionable. Other environmental risk factors, including asbestos, alcohol, and various occupational exposures, have been reviewed elsewhere (18) and will not be discussed here. Linear dose–response effects of tobacco smoking have been demonstrated consistently in both prospective and retrospective studies (19). A review of the relevant studies documents fivefold to 25-fold increased risk for smokers compared with nonsmokers (20). Higher risk for smokers of unfiltered cigarettes and a diminishing risk with increasing time since smoking cessation have also been demonstrated (21,22).

Differences by sex and by primary site have been observed both in the magnitude of the risk estimates and in the gradients of the dose response. Some studies (22,23) have reported that the risk of head and neck cancer is higher for women than for men at each successive pack-year stratum. This difference by sex has also been reported in lung cancer (24) and has been attributed to women’s greater susceptibility to tobacco carcinogens because of differences in nicotine metabolism or activity of bioactivating enzymes. Laryngeal cancers have higher smoking-associated risk estimates than do oral cavity cancers (25). These findings suggest that variable susceptibility to carcinogenic action may be both sex- and site-dependent.

Diet

Reports of the earliest epidemiologic studies of the association between dietary vitamin A and cancer did not specify the type of vitamin A consumed—preformed or provitamin A (carotenoids). A substantial body of evidence from subsequent laboratory and epidemiologic investigations indicates that dietary carotenoids are inhibitors of epithelial carcinogenesis. Inverse associations between β-carotene intake and risk of laryngeal cancer (25,26), between total vitamin A and C intake and risk of oral cancer (27), between vitamin A intake and risks of both oral and laryngeal cancer in men, and between vitamin A intake and oral cancer risk in men and women have been demonstrated (28).

Other studies (29-32) have shown that increased fruit and/or vegetable consumption is associated with a reduction in the risk of head and neck cancer. In China, Zheng et al. (29) found an inverse relationship between the risk of oral cancer and the dietary intake of total carotene, carotene from fruit and vegetables, and vitamin C. Reduced risk in this study was also associated with consumption of grapes, bananas, oranges, tangerines, peaches, and pears. Zheng et al. (30) also reported that dark green and yellow vegetables, citrus fruit, and garlic had a protective effect against laryngeal cancer. These investigators also confirmed the inverse association between cancer risk and serum levels of carotenoids (especially β-carotene) and α-tocopherol. They reported a consistent protective effect for each of the individual carotenoids, including β-carotene, cryptoxanthine, lutetin, and lycopene. In Uruguay, De Stefani et al. (31) found that eating salted meat conferred greatly increased risks of oral and pharyngeal cancers and that vegetable consumption markedly decreased these risks. A study involving 871 cases in four different parts of the United States (32) found that fruit consumption provided a dose–response protective effect against oral and pharyngeal cancers.

Except for β-carotene, the associations between diet and lung cancer are similar to those in head and neck cancer. Two large-scale intervention trials in heavy smokers, the Alpha Tocopherol, Beta-Carotene Cancer Prevention Study Group (ATBC) (33) and the β-Carotene and Retinol Efficacy Trial (CARET) (34), reported higher lung cancer rates (18% and 28%, respectively) among subjects in the β-carotene arms compared with subjects in the placebo arms. Recently published subgroup analyses of these two trials (35,36) revealed that the risk of lung cancer was highest among those individuals who continued to smoke at least 20 cigarettes per day (35) and those in the highest quintile of alcohol consumption (36). However, no data are currently available that indicate that β-carotene has a similar effect in head and neck cancer.

Only limited data exist on possible interactions between cigarette smoking and dietary factors in relation to head and neck carcinogenesis. De Stefani et al. (37) found that low fruit intake and tobacco use may act synergistically to increase laryngeal cancer risk. Zheng et al. (38) reported that the combined effects of smoking and diet were more than additive. Zheng et al. (39) also documented that the protective effects of serum carotenoids against oral and pharyngeal cancers were slightly attenuated in smokers. To the best of our knowledge, no comprehensive, prospective studies of the separate and combined effects of smoking, alcohol, and dietary factors on risk of second primary tumors of the head and neck have been conducted.

Mutagen Sensitivity as a Marker of Risk

Although smoking is the major risk factor for upper aerodigestive tract cancers, inherent host susceptibility factors also play an important role in determining risk. In vitro chromosomal analyses of sensitivity to genotoxicity are gaining approval for use in the assessment of cancer susceptibility. Hagmar et al. (40) published their cohort study of 3182 workers who had been occupationally exposed to mutagenic agents (e.g., polycyclic...
aromatic hydrocarbons (PAHs), ionizing radiation, and welding fumes). All subjects were evaluated for chromosomal aberrations at study entry. A statistically significant increase in cancer risk (relative risk $[RR] = 2.1; 95\%$ confidence interval $[CI] = 1.5-2.8$) occurred in the highest stratum of baseline aberrations. This study (40) confirms the potential value of chromosomal aberrations in peripheral lymphocytes as a marker of cancer risk.

A mutagen sensitivity assay developed by Hsu et al. (41) quantifies bleomycin-induced chromatid breaks in cultured lymphocytes to measure human risk of environmentally caused cancers. A case–control analysis (42) demonstrated that in vitro-assayed, bleomycin-induced mutagen sensitivity (analyzed either as a continuous or a dichotomous variable), after adjustment for tobacco and alcohol use, could be used as an independent risk factor for head and neck cancers (adjusted odds ratio $[OR] = 2.2; 95\% CI = 1.0-5.1$).

A recent multicenter meta-analysis (43) of results of epidemiologic studies involving patients with head and neck cancers [including Spitz et al. (42)] confirmed that mutagen sensitivity was associated with cancer risk and demonstrated that there were similarities in the size and distribution of mutagen-sensitivity values regardless of age, institution, and tobacco and alcohol use. ORs for risk of development of head and neck cancers among heavy smokers who were not mutagen hypersensitive, among heavy smokers who were mutagen hypersensitive, and among heavy smokers with hypersensitivity who also consumed alcohol were 11.5, 44.67, and 57.5, respectively.

Two hundred seventy-eight patients with upper aerodigestive tract cancers diagnosed from 1987 through 1993 were followed (44). Spitz et al. (44) reported that mutagen sensitivity is a substantial predictor of risk of multiple primary cancers. Seventeen synchronous and 11 metachronous cancers occurred in the total patient group. SPTs occurred in 16 (13.1%) of the mutagen-sensitive patients compared with 12 (7.7%) of the nonsensitive patients. Mean mutagen-induced break/cell $[b/c]$ values ($\pm$ standard deviations) for patients with SPTs and those without were 1.17 ($\pm .54$) and .93 ($\pm .44$), respectively ($P = .04$). The RR of developing SPTs was $2.67 (95\% CI = 1.22-5.79)$ for hypersensitive individuals. Sex, site of index cancer, stage, and cigarette smoking had no statistically significant influence on $b/c$ values. According to a study by Dave et al. (45), bleomycin-induced breaks in patients with head and neck cancer are not randomly distributed but occur preferentially on chromosomes 3 and 7.

The results of an analysis of the association between overall DNA repair efficiency and mutagen sensitivity in 16 established lymphoblastoid cell lines (including three head and neck cancer cell lines) indicated that reduced cellular DNA repair capacity was significantly associated with increased frequency of mutagen-induced chromatid breaks (46). Mutagen sensitivity, however, probably reflects more than just an altered repair process. The mutagen sensitivity phenotype may also reflect an inherent chromatin alteration that increases the translation of DNA damage following mutagen exposure into chromosome damage (47).

**Genetic Susceptibility**

With regard to metabolic polymorphisms, any factor that influences carcinogen absorption, distribution, or accumulation in the target tissue will also affect cancer susceptibility. The concept that environmental exposures can be modulated genetically is a possible explanation for variations in host susceptibility (48). The internal dose of tobacco carcinogens may be modulated by genetically determined polymorphisms (alterations) in the enzymes responsible for the activation and detoxification of these carcinogens.

Cytochrome P450 (CYP) 1A1, the gene that codes for aryl hydrocarbon hydroxylase (AHH), is an initiating enzyme in a multi-enzyme pathway that activates PAHs, including benz[a]pyrene, to highly electrophilic metabolites. AHH activity varies up to several thousandfold between tissues and between individuals (49) and is inducible by exposure to cigarette smoke (50).

Andreason et al. (51) reported that 26 (52%) of 50 patients with either oral or pharyngeal cancers exhibited high AHH activity compared with high AHH activity in only nine (7.6%) of 118 individuals in a normal population. Brandenberg and Kellerman (52) reported a higher prevalence of individuals who were extensive metabolizers among 90 patients with laryngeal cancer than among 230 normal control subjects. Smokers with a high AHH level had a fourfold higher risk of developing laryngeal cancer than did nonsmokers with low AHH levels. These smokers also developed cancer at younger ages and had SPTs more frequently (53).

Glutathione S-transferases (GSTs) catalyze the conjugation of glutathione to several electrophilic compounds, including carcinogenic PAHs and cytotoxic drugs. Two relevant polymorphisms of the GST family of genes, GSTM1 and GSTT1, have been reported (54,55). The absence of activity has been associated with an increased risk of GSTM1 laryngeal cancer (56). Mulder et al. (57) found that the GSTM1 phenotype was present in only four (29%) of 14 patients with oropharyngeal tumors compared with 60% of individuals in the normal population (54). There was a similar, but not statistically significant, pattern for laryngeal tumors. The risk associated with the GSTM1 null phenotype appears to be dependent on the extent of tobacco smoke exposure (58). Absence of GSTT1 similarly results in a nonconjugator phenotype (59). A study conducted by London et al. (60) revealed that the absence of GSTM1 activity also conferred an increased RR for the development of lung cancer among 356 case patients when compared with 731 control subjects ($RR = 1.29; 95\% CI = 0.94-0.77$).

We have characterized the GSTM1 and GSTT1 genotypes in 105 consecutive patients with upper aerodigestive tract cancers and in 99 age- and sex-matched control subjects (Trizna Z, Spitz M: unpublished data) (Table 1). A subset of these patients has been characterized previously by genotype (61). Analyses show univariate risk estimates of 1.37 associated with the GSTM1 null genotype and 1.43 associated with the NAT2 slow acetylator genotype, neither of which were statistically significant. There was, however, a significant association ($OR = 2.52$) with the GSTT1 null genotype. The most telling finding was elevated risks (all above threefold) when combined risk genotypes were present (Trizna Z, Spitz M: unpublished data).

Through monogenic inheritance of the NAT2 locus, the N-acetylation polymorphism segregates individuals into rapid, intermediate, and slow acetylator phenotypes. Approximately 40%-70% of Caucasians have the “slow acetylator” phenotype and are less efficient in the metabolism of agents containing...
primary aromatic amine or hydrazine groups (62). The presence of two germline copies of any of several mutant alleles of the NAT2 gene produces a slow acetylation phenotype (62,63). The NAT2 genotype is highly predictive of the acetylation phenotype and can be detected by several polymerase chain reaction- and/or restriction fragment length polymorphism-based methods (62,64). Only one study has evaluated the relationship of this genotype with head and neck cancer risk. Drozdz et al. (65) reported that slow acetylators accounted for 107 (84%) of their 128 patients with laryngeal cancer and 64 (60%) of 106 individuals in their control group (*P*< .001).

Epoxide hydrolase is another phase II enzyme that catalyzes the conjugation of PAH epoxides. Epoxide hydrolase activity appears to be under genetic control of the Ah locus (66). Cytosolic epoxide hydrolase activity in the lung tissue of recent smokers was significantly lower than that in the lung tissue of former smokers (67). Epoxide hydrolase activity was directly associated with the number of days of cessation and inversely associated with the number of cigarettes smoked per day. These data suggest that inhibition of cytosolic epoxide hydrolase activity by tobacco smoke may reduce the inactivation of carcinogenic epoxides and thereby increase susceptibility to cancer (68). Janot et al. (69) reported that epoxide hydrolase expression was significantly lower in head and neck tumors than in corresponding adjacent tissue.

**Tobacco-Related Adducts and Metabolic Activation**

Tobacco-related DNA adducts can be removed under normal circumstances by DNA repair processes or cell death. Chronic exposure to tobacco smoke, however, leads to steady-state accumulations of these adducts in target tissues. These steady-state levels generally are carcinogen dose related and predictive of tumor incidence across species. The association between tobacco-related DNA adduct levels and metabolic activation was examined in laryngeal tissue obtained from 16 patients with laryngeal cancer (70). A statistically significant association was noted between total adduct levels and metabolic activation as measured by the level of CYP1A1 activity in microsomes prepared from laryngeal tissue samples.

**Risk-Prediction Model**

Head and neck cancers generally occur in smoking-exposed individuals who are susceptible to that exposure. One susceptibility genotype or phenotype alone may not have a strong effect on cancer development in smokers, but in conjunction with other marker genes, that genotype or phenotype may increase cancer risk. Once important predictive markers and variables have been identified and validated, they can be integrated into a quantitative risk assessment model using a logistic regression analysis.

The ability to identify smokers with the highest head and neck cancer risk profiles would allow us to focus our most aggressive efforts in smoking cessation, chemoprevention, and screening on the neediest subgroup. The study of interactions between genetic susceptibility and major carcinogens can advance our understanding of carcinogenesis and guide our designs of future epidemiologic and intervention studies.

### Incidence and Distribution of SPTs

Tumor registry and retrospective studies indicate that head and neck SPTs occur at a rate of 4%-7% per year (5-7,17). Cooper et al. (71) showed that the highest cumulative risk for SPTs occurs in patients successfully treated for the earliest stage of disease. This finding reflects the shorter survival among patients with advanced stage disease. The overall annual rate of development of SPTs appears to be constant, and SPT risk does not decrease over time (71,72).

The incidence and distribution of SPTs vary according to the site of the primary tumor. The average value of SPT incidence ranges from 10% for glottic primary tumors to 40% for oropharyngeal primary tumors (73). About 60% of head and neck SPTs occur in the aerodigestive tract (lung, 30%; head and neck, 20%; and esophagus, 8%) (17). Licciardello et al. (74) found that oral cavity cancer appears to be associated more often with subsequent head and neck SPTs and laryngeal cancer appears to be associated more often with lung SPTs.

A meta-analysis of 18 published studies of SPTs associated with primary HNSCC found cumulative SPT incidences ranging from 0.061 to 0.326 (75). The highest incidences occurred in hospital-based and autopsy series, and the lowest occurred in cancer registry data. In addition to selection, classification, and follow-up biases, the major sources of variations in these 18 studies were differences in definitions of index and multiple cancers, descriptions of the temporal sequence of SPTs, and exclusion criteria used.

Early detection and successful treatment of SPTs require careful clinical surveillance. Chemopreventive agents such as retinoids are under intense investigation for the prevention of SPTs and appear to have great potential. The ability to identify high-risk subgroups will have great clinical and prognostic importance for patients with HNSCC.

A recent study of p53 protein levels by Shin et al. (76) may help in this effort. They tested for p53 levels in tumor samples from 69 patients who had received definitive local therapy for HNSCC. Patients with higher p53 protein levels had a statistically significant shorter time to recurrence (*P* = .47; 95% CI = 0.99-4.26), a shorter time to the development of an SPT (*P* = .03; 95% CI = 1.07-7.00), and a statistically significant decrease in overall survival (*P* = .0002; 95% CI = 1.30-4.89). Koch et al. (77) used direct sequence analysis to evaluate p53 mutational status with respect to locoregional treatment failures in 110 patients with head and neck cancer. They noted a significantly increased risk (RR = 2.2; 95% CI = 1.2-4.1; *P* = .02) of local

### Table 1. Polymorphisms of glutathione S-transferase (GST) MU, THETA, and NAT2 genotype in head and neck cancer*,

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1 null</td>
<td>1.37</td>
<td>0.8-2.4</td>
</tr>
<tr>
<td>GSTT1 null</td>
<td>2.52</td>
<td>1.4-4.7</td>
</tr>
<tr>
<td>NAT2 slow</td>
<td>1.43</td>
<td>0.3-2.6</td>
</tr>
<tr>
<td>Combined genotypes</td>
<td></td>
<td></td>
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<tr>
<td>GSTM1 + GSTT1</td>
<td>3.46</td>
<td>0.5-7.8</td>
</tr>
<tr>
<td>GSTM1 + NAT2 slow</td>
<td>3.30</td>
<td>1.1-6.6</td>
</tr>
</tbody>
</table>

*n* = 105 patients with upper aerodigestive tract cancers; 99 control subjects. †Risk estimates for head and neck cancer by select genotype (from unpublished data by Z. Trizna and M. Spitz, with permission).
or regional recurrence in those patients whose tumors demonstrated mutations of the p53 gene.

**Retinoid Biology, Pharmacology, and Metabolism**

**Biology and Pharmacology**

Retinoids have complex biologic effects, including modulations of differentiation, proliferation, and apoptosis, within both normal and neoplastic tissues. This complexity is not only a function of the diversity of the retinoid ligands but of the diversity of the nuclear receptors that mediate their activity. Different natural retinoids can activate different retinoid receptors expressed in different cell types and thus differentially regulate gene expression (78,79).

There are two classes of nuclear retinoid receptors—retinoic acid receptors (RARs) and retinoid X receptors (RXRs). There are three subclasses: alpha, beta, and gamma, each divided further into a large number of isoforms produced through different promoter usage and alternative splicing of receptor transcripts (78,79). Specific isoforms of RARs and RXRs appear to have different functions and to be expressed differently in various tissues (78).

Nuclear retinoid receptors function either as heterodimers or homodimers. RXRs can form homodimers or heterodimers by binding with RARs or a host of other receptors, such as those for vitamin D, thyroid hormones, and prostaglandin-J2. RARs form only heterodimers and only with RXRs. Therefore, RXR ligands are more versatile than RAR ligands in activating multiple retinoid and other pathways, thereby triggering the downstream expression of various genes (78,79).

Natural retinoids are nonselective, pan-receptor activators, meaning they cause a wide spectrum of physiologic effects. Subtle differences in the structure of the cleft where retinoids bind to receptors have permitted the development of receptor-specific synthetic retinoids. Since different receptors may mediate different effects, this specificity, or selectivity, can have important clinical implications for enhancing the desired effects and reducing the undesired effects. For example, a retinoid specific only for RAR-β can be active in certain squamous carcinoma cells, but because it does not bind to RAR-γ, the toxic effects mediated by that receptor are avoided. It is hoped that these specific retinoids can overcome the substantial and limiting toxicity caused by the widely used natural retinoids, all-trans-retinoic acid (ATRA) and its two isoforms, 9-cis-retinoic acid (9cRA) and 13-cis-retinoic acid (13cRA), that have been used to date in clinical trials (80).

Retinoid receptors can affect gene transcription either directly or indirectly. This phenomenon is illustrated by the receptor interaction with activator protein-1 (AP-1) (81,82). This transcription factor is an important regulator of proliferative and inflammatory responses (83).

Furthermore, recent work by Kamei et al. (84) has demonstrated that retinoid-mediated inhibition of AP-1 activity appears to be the result of competition between nuclear retinoid receptors bound to their receptors and AP-1 for a coactivator that is necessary for effective ligand-dependent gene activation by nuclear receptors. This coactivator is found in limited quantities in the cell (84).

The ability of retinoic acid to inhibit abnormal squamous differentiation has important implications for both prevention and therapy (80). This activity increases neoplastic tissue sensitivity to cytotoxic drugs (chemotherapy) and may help reverse the underlying molecular alterations associated with carcinogenesis (chemoprevention). Retinoid inhibition of squamous differentiation may be mediated by AP-1 (85), which is known to drive the squamous differentiation process. Several squamous differentiation genes contain AP-1 elements in their promoter regions (85).

AP-1-selective synthetic retinoids also can be potent antiproliferative agents in a number of tumor-derived cell lines (81,82,85). These agents do not appear to affect differentiation, which has been linked to retinoid toxicity.

An area of critical importance in the field of retinoid chemoprevention is drug resistance. De novo retinoid resistance occurs in 40% of oral premalignant lesions and develops over time in many lesions that responded previously to retinoid treatment (7). The mechanisms that underlie retinoid resistance have not yet been clearly elucidated (78-85). This has resulted in increased interest in the evaluation of novel retinoids, such as 4-N-(hydroxyphenyl)retinamide (4-HPR) (87), which does not appear to bind any of the retinoid receptors (86) but is a potent in vitro inducer of apoptosis (86).

Other groups have focused on the use of retinoids as potential chemosensitizing agents. Shalinsky et al. (87) combined cisplatin with 9cRA in human oral squamous carcinoma xenografts in nude mice, demonstrating enhanced antimtumor efficacy bordering on synergism.

**Metabolism**

The four factors that determine the levels of various retinoic acids within either normal or neoplastic cells are as follows: the level of serum all-trans-retinol (vitamin A), the cellular uptake of serum retinol, the activity of the cellular enzymes that oxidize retinol to retinoic acid, and the activity of the enzymes that catabolize retinoic acid (88). The very low level of retinoic acid in normal plasma is considered to be too low to support biologic processes.

Abnormally low intracellular retinoic acid level is directly associated with the development of cancer. Perturbations of retinoid metabolism rather than a vitamin A-deficient diet generally cause deficient serum vitamin A levels (88).

In a provocative study, Xu et al. (89) evaluated the mechanism of decreased RAR-β expression in oral premalignant lesions (OPLs). They theorized that RAR-β suppression could result from a decrease in the cellular level of retinoids, since they had previously demonstrated that retinoids enhance transcription of the RAR-β gene (90). They therefore evaluated the binding of a monoclonal antibody against ATRA to normal tissue and OPLs. While all seven normal specimens stained positive with the antibody, only 20 of 43 OPLs stained positive. Of the 24 specimens available for evaluation both before and after 3 months of 13cRA treatment in vivo, anti-RA monoclonal antibody bound to 22 specimens (92%) after treatment, having bound to only 10 (42%) before treatment. Similarly, RAR-β expression increased from seven (29%) of 24 specimens before treatment to 21 (88%) of 24 specimens after treatment. These results and the strong association between anti-RA monoclonal antibody binding and RAR-β expression suggest that anti-RA
monoclonal antibody binding effectively reflects the level of ligand present in the tissues.

Defects in retinoid metabolism may stem from defective levels or functions of metabolizing proteins and enzymes or from aberrations in the retinoid signaling pathway itself. High levels of cellular retinol-binding protein (promoting retinol esterification and storage over oxidation to retinoic acid) or defects in the activity of retinol and retinal dehydrogenase or of their cofactors nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate may impede normal vitamin A metabolism (88).

Studies of retinoid signaling have revealed that an RAR gene alteration has been linked to malignant transformation in acute promyelocytic leukemia and hepatitis-linked hepatocellular carcinoma (78). Defective retinoid receptors directly alter endogenous retinoid regulation of gene expression. Intact retinoid receptors also can fail to activate downstream genes, as illustrated by lung cancer studies showing normal retinoid activation of receptors but a lack of retinoid-responsive gene transcription (91).

The most effective way to compensate for aberrant retinoid metabolism in transformed cells may be to deliver biologically active retinoids directly to them, thus bypassing the physiologic pathways of normal retinoid metabolism. This is the basic strategy of current retinoid pharmacotherapy with ATRA, 9cRA, and 13cRA, which diffuse across plasma membranes and gain direct access to intracellular binding proteins and receptors (88).

**Clinical Trials**

Several randomized chemoprevention trials involving retinoids and/or β-carotene are ongoing or have been completed in the head and neck (Tables 2 and 3). Retinoids are by far the most studied and active chemopreventive agents in head and neck cancer. Specific retinoids studied include retinol, retinyl palmitate, ATRA, 13cRA, etretinate, and 4-HPR (Fig. 2). Retinoid chemoprevention strategies that have focused on head and neck cancer involve either attempts to reverse OPLs or to prevent the development of SPTs.

**Oral Premalignancy Trials**

OPLs include both leukoplakia and erythroplakia. Small hyperplastic leukoplakia lesions have a 30%-40% spontaneous regression rate and a less than 5% risk of malignant transformation. Erythroplakia and dysplastic leukoplakia lesions have a less than 5% rate of spontaneous regression and a 30%-40% risk of developing oral cancer (5). This high-risk, diffuse, and multifocal disease accounts for approximately 10%-15% of all OPLs and is rarely controlled adequately with local therapy involving surgery or radiation therapy. Patients with OPLs often develop squamous cancers at distant sites in the upper aerodigestive tract as well as in the oral cavity. Therefore, the trials focusing on the reversal of oral premalignant lesions may prove to be useful in identifying agents with the potential of preventing aerodigestive tract cancers.

Epidemiologic studies in the late 1970s found that supplemental β-carotene and retinol were able to statistically significantly reduce the frequency of oral micronuclei (80), which are fragments of extranuclear DNA thought to represent a nonspecific but quantifiable assessment of genetic damage in populations at high risk for oral cancer, including tobacco chewers and betel nut chewers. Seven subsequent trials have been conducted to investigate the effects of supplemental β-carotene, alone or in combination with other agents, on the progression of oral leukoplakia. Five were nonrandomized studies achieving response rates of 44%-71% (80). These uncontrolled trials results are tempered by leukoplakia’s 30%-40% spontaneous regression rate, different study response criteria, and the absence of any readily apparent dose–response relationship (80).

Two placebo-controlled trials of β-carotene in oral leukoplakia have been conducted. Stich et al. (92) reported that combined β-carotene plus retinol, β-carotene alone, and placebo produced complete response rates of 27.5%, 14.8%, and 3.0%, respectively. Stich did not report partial remission rates. A trial in Uzbekistan (93) used 6 months of therapy with the combination of retinol, β-carotene, and vitamin E. These investigators demonstrated a significant reduction in the prevalence OR of oral leukoplakia (OR = 0.62; 95% CI = 0.39-0.98). However, while the risk of progression or no change versus regression was also reduced 40% by this combination, this finding was not statistically significant (OR = 0.60; 95% CI = 0.23-1.63).

Benner et al. (94) conducted a single-arm phase II study of α-tocopherol in 58 patients with oral leukoplakia. Forty-three patients completed 24 weeks of therapy, 20 (47%) having clinical responses and nine (21%) having histologic responses. The results of five randomized trials (95-100) involving retinoids in patients with OPLs have been reported (Table 2); one of these trials included a β-carotene arm (96). Earlier positive single-arm trials had small sample sizes and were unblinded (101,102). In 1986, Hong et al. (95) reported the results of their prospective, randomized, double-blinded clinical trial of high-dose 13cRA (1-2 mg/kg per day) in oral leukoplakia. Clinical responses occurred in 16 (67%) of the 24 patients in the 13cRA group and in two (10%) of 20 patients in the placebo group (P = .002). The histopathologic improvement (reversal of dysplasia) rate also was higher in the retinoid arm (54% versus 10%; P = .01). Major trial problems included substantial toxicity and a high rate of relapse (>50% within 2-3 months of discontinuing therapy).

In a subsequent study, Lippman et al. (96) investigated a low dose of 13cRA to address the toxicity and relapse problems of the first randomized trial. Patients received a 3-month induction course of high-dose 13cRA (1.5 mg/kg per day), followed by a 9-month maintenance treatment with either low-dose 13cRA (0.5 mg/kg per day) or β-carotene (30 mg per day). Induction produced a high rate of response (55%; 95% CI = 42%-67%). During the maintenance phase, only two (8%) of the 24 patients in the low-dose 13cRA maintenance group had progression of leukoplakia versus progression in 16 (55%) of the 29 patients on β-carotene maintenance (P < .001). Low-dose 13cRA was well tolerated, with no patients dropping out of the trial during the maintenance phase because of 13cRA toxicity.

Stich et al. (97) compared vitamin A (200000 IU/week orally for 6 months) with placebo in 54 tobacco–betel nut chewers in India who had well differentiated oral leukoplakia. Complete remission occurred in 12 (57.1%) of the 21 patients receiving vitamin A versus one (3%) of the 33 control subjects.

Two randomized trials of synthetic retinamides, 4-HPR and
4-(hydroxycarbophenyl)retinamide, also have been conducted in oral premalignancy. Han et al. (98) randomly assigned 61 patients to receive either 4-(hydroxycarbophenyl)retinamide (40 mg/day) or placebo. The retinamide was effective in reversing OPLs (Table 2). In 1988, Chiesa et al. (99) began a randomized trial in Milan to evaluate the efficacy of systemic 4-HPR maintenance therapy following complete laser resection of OPLs. The most recent update of this study (100) included data from 153 patients who had been randomly assigned to receive either 4-HPR (200 mg/day for 52 weeks) (n = 74) or no intervention (n = 79). Treatment included a 3-day drug holiday at the end of each month to avoid the night blindness caused by 4-HPR lowering serum retinol. Twenty-one treatment failures (eight recurrences, 12 new lesions, and one cancer) occurred among the patients in the control group versus nine failures (seven recurrences, two new lesions, and no carcinomas) among the patients in the 4-HPR group.

Subsequent research efforts have focused on the combination of high-dose 13cRA, α-tocopherol, and interferon alfa (IFN α) for the treatment of patients with moderate and severe dysplasia. The rationale for the use of this combination is predicated on a series of 257 untreated patients with leukoplakia who were followed by Silverman et al. (103). They found that the malignant transformation rate at 8 years was 17.5%. However, when they evaluated only those leukoplakia patients with dysplasia on the initial biopsy, they found that the transformation rate at 8 years of follow-up increased to 34.6%. None of the dysplastic lesions followed by Silverman et al. (103) resolved spontaneously, thus confirming the theory that severe dysplasia or carcinoma in situ has a very high rate of malignant transformation into invasive squamous cell carcinoma. Although two previous studies (92,93) established the activity of 13cRA in early oral premalignancies, such as atypical hyperplasia and mild dysplasia, this activity was not demonstrated in advanced premalignant lesions that included moderate to severe dysplasia. A study (104) recently completed at The University of Texas M. D. Anderson Cancer Center that used high-dose 13cRA and α-tocopherol demonstrated activity in advanced premalignant lesions resistant to retinoid alone and also suggested a reduction of the retinoid-associated toxic effects. Other studies (105,106) of 13cRA in combination with IFN α have shown enhanced therapeutic effects in patients with skin or cervical cancer. This work is based on supportive in vitro
and in vivo work (107,108). Currently, a phase II clinical trial is being conducted at The University of Texas M. D. Anderson Cancer Center to explore the potential use of 13cRA, α-tocopherol, and IFN α as biochemoprevention therapy in advanced, dysplastic premalignant lesions of the upper aerodigestive tract.

Second Primary Tumors

The high likelihood of SPTs following treatment of early HNSCC makes this disease an excellent model for the chemoprevention of SPTs. Hong et al. (109) conducted a randomized, double-blinded, placebo-controlled trial of high-dose 13cRA as adjuvant therapy following curative surgery and/or radiation therapy of primary HNSCC. One hundred three patients were randomly assigned to receive either high-dose 13cRA (50-100 mg/m² per day) or placebo for 12 months. After a median follow-up of 32 months, SPTs developed in significantly fewer 13cRA-treated patients (4%) than in patients receiving placebo (24%) (P = .005). A total of 14 SPTs occurred, with 13 (93%) located in the tobacco smoke-exposed field of the upper aerodigestive tract, lungs, and esophagus. The limitations of this study were the substantial toxicity of high-dose 13cRA (one third of the retinoid-treated patients required dose reductions or discontinuation of therapy) and a lack of impact on recurrence or overall survival. These data were reanalyzed after a median follow-up of 4.5 years (110). Retinoid-treated patients continued to have significantly fewer total SPTs—seven (14%) in the 13cRA arm and 16 (31%) in the placebo arm (P = .042). When only SPTs that developed in the tobacco-exposed field of the upper aerodigestive tract or lungs were considered, the results were even more impressive—SPTs occurred in three of 49 13cRA-treated patients and in 13 of 51 assessable patients receiving placebo (P = .008). These results were provocative because they suggested that the chemopreventive effect of 13cRA persisted for approximately 3 years after the completion of therapy. However, this effect disappeared subsequently, as demonstrated by the fact that the SPT rates in both arms were equivalent from that point onward.

Pastorino et al. (111) conducted a randomized trial of retinyl palmitate (300,000 IU/day for 12 months) to prevent SPTs in patients previously treated for primary stage I non-small-cell lung cancer. The retinoid was generally well tolerated, with more than 80% of the patients compliant with the treatment. Eighteen patients in the retinyl palmitate group developed SPTs compared with 29 patients in the control arm. A reduction in tobacco-related SPTs occurred—only 13 SPTs occurred in patients in the retinyl palmitate arm versus 25 SPTs in patients in the control arm. Also, time to development of a tobacco-related SPT statistically significantly favored the retinoid arm (P = .045).

Bolla et al. (112) used etretinate to prevent SPTs in patients with prior squamous cell cancers of the oral cavity or oropharynx. Patients were randomly assigned to receive either the retinoid (50 mg/day for 1 month, followed by 25 mg/day for 24 months) or placebo. After a median follow-up of 41 months, no SPT reduction was seen in the retinoid arm. The two study arms were equivalent both in the occurrence of SPTs and relapse of the initial cancer. This prospective trial, however, confirmed the high rate of head and neck SPTs seen in the earlier trials of Hong et al. (109,110). SPTs occurred in 57 (18%) of the 316 patients enrolled in the trial conducted by Bolla et al. (112), with 45 (79%) of these SPTs occurring in the upper aerodigestive tract, lungs, and esophagus (Table 3).

To resolve the conflicting results of the two previous head and neck SPT trials, in 1992, investigators at the University of Texas M. D. Anderson Cancer Center (113) launched the largest head and neck cancer chemoprevention trial ever attempted through the intergroup mechanism. Target accrual is 1302 patients with stage I or II HNSCC previously definitively treated with radiation therapy or surgery. An 8-week run-in period is being used to improve compliance. After run-in, patients are randomly assigned to receive either 3 years of 13cRA (30 mg/day) or placebo. To date, toxicity data reveal that the dose of 13cRA, lower than in the earlier trial of Hong et al. (109), has been relatively well tolerated, with an overall noncompliance rate of approximately 21% at 2 years. Accrual to this study is expected to be completed in June 1998. When completed, this carefully designed trial should define the role of 13cRA in preventing head and neck SPTs.
Translational Studies of Biomarkers as Intermediate End Points

Translational research is critical for developing and testing new chemopreventive agents. The OPL is an excellent model for the conduct of translational retinoid studies. Oral premalignant lesions can be relatively easily monitored and sampled, are associated with the development of frank cancers throughout the aerodigestive tract, and have responded to retinoids in definitive clinical trials. These attributes make the retinoid–OPL system a paradigm for clinical laboratory collaboration in biomarker research (114).

On the basis of suggestive findings of studies of nuclear retinoid receptors in squamous tumors and surrounding tissue (115), Lotan et al. (90) evaluated biopsy specimens of 52 patients with OPLs for the expression of RARs and RXRs both before and after 13cRA treatment. These receptor findings were then compared with receptor findings in normal control subjects. All normal specimens contained RAR-β messenger RNA (mRNA), but only 21 (40%) of the 52 pretreated OPLs had detectable RAR-β mRNA levels at baseline. After the patients on the clinical trial completed 3 months of treatment with 13cRA (96), 35 (90%) of the 39 OPL specimens available for evaluation expressed RAR-β. This statistically significant improvement of RAR-β expression after 13cRA treatment (P < .001 by McNemar’s test) demonstrates both that RAR-β is selectively down-regulated in OPLs and that treatment with 13cRA can up-regulate RAR-β in these lesions (Fig. 3).

Lippman et al. (116) subsequently used the OPLs–retinoid system to study p53 protein accumulation in relation to RAR-β expression and response to retinoid. p53 protein accumulation was statistically significantly associated with resistance to retinoid and with a lack of RAR-β up-regulation. 13cRA did not attenuate p53 accumulation in OPLs that overexpressed p53 protein in this study.

Conclusion

Basic scientists, epidemiologists, and clinicians are working collaboratively to better understand, prevent, and treat HNSCCs. Advances in our understanding of the fundamental processes involved in multistep and field carcinogenesis are providing direction to clinical researchers for the design of chemoprevention trials. Molecular studies of genetic alterations, such as LOH and p53 gene mutations, have been especially valuable in this regard. Epidemiologic advances in understanding the interactions between host genetic susceptibility (e.g., mutagen sensitivity) and environmental risk factors (primarily cigarette smoke) are bringing us closer to the end point of designing an accurate risk prediction model for HNSCC. This model will help focus our most aggressive prevention efforts on smoking cessation, screening, and chemoprevention within the highest-risk subgroup of smokers and other exposed individuals.

In clinical research, great strides have been made in defining the role of active retinoids, particularly 13cRA, in the reversal of OPLs and the prevention of head and neck SPTs. An ongoing, large, multi-institutional phase III trial of 13cRA is expected to definitively establish whether this retinoid should become the standard of adjuvant care for patients successfully treated for head and neck cancers. Clinical trials in SPT prevention and in the retinoid–OPLs model are ongoing in a translational relationship with laboratory studies of the biologic and molecular effects of retinoids in head and neck carcinogenesis.

Investigators must continue to search for novel, effective regimens to control this deadly family of cancers. Translational chemoprevention trials of retinoids continue to show promise for further advances in the prevention and treatment of HNSCC, thus providing a paradigm of research methodology for the study of other chemopreventive agents.

Table 3. Randomized chemoprevention trials in patients with aerodigestive tumors to prevent second primary tumors involving retinoids and/or β-carotene*

<table>
<thead>
<tr>
<th>Investigator(s) (reference No.)</th>
<th>Year</th>
<th>Patient population</th>
<th>No. of patients</th>
<th>Median follow-up, mo</th>
<th>Agent(s)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hong et al., Benner et al. (109,110)</td>
<td>1990, 1994</td>
<td>HNSCC</td>
<td>103</td>
<td>54</td>
<td>13cRA (50–100 mg/m²) for 12 mo versus placebo</td>
<td>14% SPTs versus 31% (P = .005)</td>
</tr>
<tr>
<td>Pastorino et al. (111)</td>
<td>1993</td>
<td>NSCLC</td>
<td>307</td>
<td>46</td>
<td>Retinyl palmitate (300 000 IU) for 12 mo versus placebo</td>
<td>8.5% SPTs versus 18.8% SPTs (P = .05)</td>
</tr>
<tr>
<td>Bolla et al. (112)</td>
<td>1994</td>
<td>HNSCC</td>
<td>316</td>
<td>41</td>
<td>Etretinate (50 mg/day) for 1 mo followed by 25 mg/day for 24 mo versus placebo</td>
<td>No difference (25% in both groups)</td>
</tr>
</tbody>
</table>

*HNSCC = head and neck squamous cell carcinoma; NSCLC = non-small-cell lung cancer; 13cRA = 13-cis-retinoic acid; SPT = second primary tumor.
of new agents, such as α-tocopherol, IFN-α, and retinamides, and their integration into combined-modality approaches.

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which occurs less frequently than binding to normal tissue, increases after 13-cis-RA treatment in vivo and is related to RA receptor beta expression. Cancer Res 1995;55:5077-11.


Notes

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