*Cancer and Metastasis Reviews* **15**: 53–76, 1996.


# Molecular and cellular biomarkers for field cancerization and multistep process in head and neck tumorigenesis

Vali A. Papadimitrakopoulou, Dong M. Shin and Waun K. Hong
*Department of Thoracic/Head and Neck Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA*

*Key words:* field cancerization, multistep tumorigenesis, biomarkers, aerodigestive tract epithelium

**Summary**

One way to explain the development of head and neck cancer is through the theories of field cancerization, i.e., the exposure of an entire field of tissue to repeated carcinogenic insult, and multistep process, i.e., development of multiple cancers in a predisposed field through a series of recognizable stages. Recent molecular genetic studies of histologically normal and premalignant epithelia of high-risk subjects and studies of malignant tumors in aerodigestive tract epithelia have identified a continuum of accumulated specific genetic alterations that possibly occur during the clonal evolution of tumors, namely, during the multistep process. Second primary or multiple primary tumors arise in the same fields as independent clones, with similar but unique molecular genetic and/or cellular alterations. Consequently, the assessment of these genetic and phenotypic alterations has been integrated into clinical chemoprevention trials in an effort to identify biomarkers that are also risk predictors and intermediate end points. This review covers candidate biomarkers of the processes of field cancerization and multistep tumor development in aerodigestive tract epithelia, including general and specific genetic markers, proliferation markers, and squamous differentiation markers.

## Introduction

Head and neck cancers account for an estimated 42,000 new cases of cancer and 12,000 deaths per year in the United States, roughly 4% of all new cancer cases and 2% of cancer deaths annually [1]. The worldwide incidence of this malignancy, approximately 500,000 cases per year, has remained unchanged for over two decades [2, 3].

Over the last 20 years, diagnosis and management have improved through combined efforts in sugery, radiotherapy, and chemotherapy and through tobacco cessation programs, but long-term survival rates have improved only marginally and are among the lowest of the major cancers [4, 5]. In addition, the residual cosmetic and functional effects of definitive local therapy often debilitate and reduce the quality of life of these patients.

New strategies are clearly needed to help control head and neck cancer. One of the most important approaches under investigation is chemoprevention, the use of specific natural or synthetic agents to suppress, reverse, or prevent carcinogenesis [6–9]. This approach can be used in the general population, in subjects with increased cancer risk due to inherited or acquired factors, and in patients cured of primary tumors but at risk for second primary tumors [10–15]. The major obstacle, however, in designing trials for such interventions is the extremely distant end point, namely cancer incidence. Reaching that end point is expensive and requires many subjects, and long-term follow-up. One obvious so-

lution is to identify intermediate end points using biomarkers associated with specific stages of the carcinogenic process [16–18].

Research so far into head and neck tumorigenesis has been based on two theories: field cancerization and multistep process. The phenomenon of field cancerization is demonstrated in tissue samples from different sites in the aerodigestive tract, a 'field' in which tissue is exposed to the same carcinogenic insult. The frequent presence of a variety of premalignant and malignant lesions at these sites reflects the multistep process of tumorigenesis. Research efforts have identified many potential cellular and molecular biomarkers [19–23] and set the stage for future clinical studies [24].

Strictly, these candidate biomarkers should be validated through clinical chemoprevention trials before being used as risk assessment or intermediate end points. Although this could take many years and require many subjects, promising currently available biomarker candidates should be incorporated into current and future chemoprevention trials. The most useful chemopreventive agents, a structurally heterogeneous and mechanistically diverse group, could be selected on the basis of modulation of biomarkers, with an eye to establishing combinations that effectively inhibit different phases of the carcinogenic process, as reflected in the biomarkers. Also, certain biomarkers might be used to select high-risk populations for chemoprevention trials. In the long term, panels of intermediate end point biomarkers may replace cancer incidence as the major end point of phase III chemoprevention trials.

## Field cancerization

The concept of field 'cancerization' was proposed in 1953 by Slaughter *et al.* [25], who hypothesized that the entire epithelial surface of the aerodigestive tract is exposed to common carcinogens and has an increased risk of cancer development. They also postulated that multiple primary epithelial cancers could arise independently within the aerodigestive tract following prolonged exposure to carcinogens. Patients with head and neck squamous cell carcinoma, or those at high risk for the development of this cancer, commonly have a history of exposure to cigarette smoke and are likely to have multiple premalignant or malignant lesions clinically and histologically evident within the same epithelium of the aerodigestive tract. This theory is supported by clinical, histopathological, and recent molecular biological evidence.

### *Clinical evidence*

Head and neck squamous cell carcinomas usually occur in tissues heavily exposed to carcinogen. Epidemiologic and, more recently, molecular data show that tobacco products increase tumor risk in the exposed field. The tumors can occur throughout the aerodigestive tract, and the risk of tumor development correlates with the duration and extent of carcinogenic exposure. Moreover, concurrent exposure to other etiologic agents (e.g., alcohol) can further increase the risk within the exposed field.

Interestingly, a significant percentage of patients initially cured of their cancers go on to develop second primary tumors (SPTs). These SPTs, whether synchronous or metachronous, develop at a constant rate (4–7% of treated patients per year), usually in the field at risk: the head and neck, the upper two thirds of the esophagus, and/or the lung. Patients with early-stage disease are very prone to SPTs since their risk for metastatic disease and local relapse is greatly decreased by their definitive primary treatments [26–31]. There is much evidence to support this. A retrospective survey of 235 laryngeal cancer patients followed for a median of 10 years revealed that 21% developed SPTs [32]; most of the SPTs occurred in the aerodigestive tract, 18% occurred in the head and neck, and 4% occurred in the lung. Furthermore, the SPTs occurred most often in patients diagnosed with early-stage disease. Cooper and colleagues, in a report from the Radiation Therapy Oncology Group's study of 928 patients who received radiotherapy for their primary tumors and were then followed for 8 years, observed that 25% developed SPTs. Nearly 60% of those SPTs occurred in the aerodigestive tract, mostly in patients with the earliest primary tumors. In analyz-

ing the incidence, anatomic distribution, and survival impact of SPTs of the aerodigestive tract with respect to specific head and neck cancer sites [33], Licciardello *et al.* found a 10–40% incidence of metachronous tumors and a 9–14% incidence of synchronous SPTs (mostly lung, head and neck, and esophageal). These SPTs unquestionably influenced subsequent survival and proved to be the major threat to long-term survival after successful therapy of early-stage head and neck cancer. Finally, Vikram found that the risk of SPTs at 4 years after initial treatment exceeds the risk of relapse [34].

*Histopathological evidence*

In addition to Slaughter's clinical observations in the late 1950's, Auerbach and colleagues extensively examined paraffin sections of lungs from over 100 dying patients with and without lung cancer [35]. They divided the tracheobronchial tree into 208 sections and histologically analyzed the paraffin sections. Some epithelial change was evident in 90–100% of sections derived from most of the light smokers, all of the heavy smokers, and all patients with lung cancer, ranging from loss of cilia and basal cell hyperplasia to carcinoma-*in-situ*. These changes were present throughout the lung field, and the degree of change correlated with the extent of tobacco exposure. Additional evidence comes from the mouse skin model, a good model for the progression of intraepithelial neoplasia [36]. In this model, uniform exposure of the entire epithelial surface to environmental carcinogens leads to multiple, typically monoclonal papillomas derived from a single stem cell of the epidermal proliferative unit. Two other examples of carcinogen exposure throughout the epithelium of an organ are exposure of the skin to sunlight and of the colon to fatty acids (in the colon, areas of the crypt epithelium develop hyperplastic and then dysplastic changes in the epithelium covering the adenomatous polyps). The result is diffuse injury and a higher than normal risk of multiple primary tumors [37, 38].

*Molecular biological evidence*

More proof for the notion of field cancerization comes from molecular biology and the characterization of molecular events occurring in tumor fields. Studies by Sozzi *et al.* [39] and Lee *et al.* [40] of short-term cultures of normal lung cells from patients with lung tumors revealed multiple chromosomal changes including rearrangements, aneusomy (e.g., trisomy 7), and deletions (e.g., 3p and 17p deletions). The frequency of the changes were significantly higher in patients with multiple primary tumors of the aerodigestive tract. Our group has also characterized the molecular events in lung tumor fields, using the technique of premature chromosome condensation to visualize interphase chromosomes of cytologically normal lung cells obtained at pneumonectomy for lung tumors [41, 42]. The lung cells away from the tumor site harbored genetic changes, including varying numbers of chromosomes per cell (suggesting genomic instability) and karyotypic structural changes. In some cases, these changes were shared by the tumor, suggesting that specific genetic changes might enhance the survival of damaged cells or that the tumor and the cells from normal tissue harboring the same changes have a common clonal origin. In other cells, distinct changes were noted, suggesting that genetic damage continues to accumulate in the normal tissue, increasing the risk that important genetic loci will be altered and that multiple clones and multiple primaries will develop [42]. Furthermore, using nonisotopic *in situ* hybridization, specific chromosome probes were applied in paraffin-embedded head and neck tissue sections containing the whole spectrum of premalignant and malignant lesions that all showed chromosomal abnormalities and will be discussed in detail later in this chapter [43].

Probes for specific genetic changes involving oncogenes and tumor suppressor genes in aerodigestive tract tumors have also been used to characterize the rest of the genetic changes in the field. For instance, Rabbits and colleagues reported the same molecular changes in regions of bronchial dysplasia and adjacent invasive carcinoma of the lung [44]. Gazdar *et al.*, using polymerase chain reaction methodologies and microsatellite primers and

probes, revealed a variety of clonal changes including *ras* mutations and allelic losses of chromosomes 9p and 3p in premalignant epithelial regions from lung tissues of cancer patients [45–47].

Finally, the observation that specific molecular events (i.e., *p53* mutations, deletion associated with loss of heterozygosity) usually differ between multiple primary tumors [48] and between different premalignant foci supports the notion that genetic changes occur independently throughout the aerodigestive tract, leading to multifocal tumor formation.

## Multistep tumorigenesis

In multistep tumorigenesis, the accumulation of genetic damage results in phenotypic changes, i.e., dysfunctional regulatory processes, that affect cell growth, differentiation, cell loss, and function. This theory dovetails with the increasingly apparent notion that cancer develops in a stepwise process involving genetically altered cell populations. Some of the altered cell populations may spawn 'new' genetically altered cells that are more likely to evolve into different neoplastic foci. Indeed, the carcinogenic process seems to be one of cellular evolution based upon the repeated selection of genetically altered cells whose chances of becoming cancerous increase as the system evolves through the precancerous stage and clonal expansion [49].

Early genetic lesions may be present in this affected field and be targets of further complex and multiple genetic changes during the pathogenesis of aerodigestive tract tumors. Although the earliest genetic changes may involve both altered differentiation and proliferation, continuing genetic changes are likely to accompany the proliferative changes as carcinogenesis progresses. The accumulation of genetic damage in the exposed tissue, which may drive carcinogenesis, culminates in the events necessary for cell proliferation and transformation of the cells from genetically altered but histologically normal to frankly malignant. The rate of accumulation of genetic damage reflects the degree of carcinogen exposure, the inherent sensitivity of the individual, and the degree of tissue damage. However, although the whole field presumably accumulates genetic damage, only a few malignant foci eventually develop into carcinomas. One theory is that only those cells that take the right genetic 'hits' at the premalignant stages can become malignant. These 'hits' may be activation of proto-oncogenes and inactivation of tumor suppressor genes. Indeed, their correlation with histopathologic progression has led to the colorectal cancer model of molecular progression [50]. In this model, the progression from colorectal adenoma to carcinoma involves *ras* oncogene mutations and the loss of tumor suppressor genes on 5q, 17p, and 18q, with each genetic alteration being followed by clonal expansion. A similar but unique molecular genetic model is emerging for head and neck tumorigenesis.

### *Clinical and histological evidence*

From a clinical point of view, tumors of the aerodigestive tract often follow, but do not necessarily originate from abnormal premalignant lesions in the field at risk (i.e., oral leukoplakia/erythroplakia). Indeed, the presence of such lesions often implies a higher tumor risk somewhere in the field. Premalignant oral lesions occur in up to 30% of oral squamous cell carcinoma cases [51, 52]. These premalignant lesions often have hyperplastic and dysplastic phenotypes, both of which can progress to invasive cancer [38, 53–54]. For instance, a study that followed 257 oral leukoplakia patients for 7.2 years saw the development of squamous cell carcinoma in 17.5% of cases. It also revealed that dysplastic lesions had a ten-fold higher transformation and a ten-fold lower spontaneous regression rates than hyperplastic lesions [55]. Other clinical examples of the multistep process are the development of esophageal cancer from Barrett's esophagus, of lung cancer from bronchial metaplasia, and of invasive transitional cell carcinoma in the bladder from intraepithelial neoplasia outside the aerodigestive tract areas.

Thanks to the identification of specific genetic changes that drive the neoplastic process, a model for head and neck squamous cell carcinoma tumorigenesis is now evolving that should be useful in

early detection, intervention, and prognosis. These genetic changes and their relevance to both field cancerization and multistep tumorigenesis will be described in detail in the following biomarker section.

## Biomarkers

Recent technical advances, wide applications of molecular biology, and increasing emphasis on translational research have revealed much about the determinants of malignant progression. A panel of potential biomarkers identified to date will be assessed individually and details are presented by specific category in Table 1.

*Table 1.* Biomarker candidates

| | |
|---|---|
| General genomic markers | |
| Nuclear abberrations (micronuclei) | |
| Chromosomal alterations | |
| Specific genomic markers | |
| Proto-oncogene alterations | |
| | Ras (H-, K-, N-ras) |
| | ErbB1/EGFR |
| | PRAD-1/Cyclin D1 |
| Tumor suppressor genes | |
| | p53 |
| | p21 (WAF1/CIP1) |
| | retinoblastoma gene (rb) |
| | MTS-1 (p16) |
| | 3p (unidentified) |
| Proliferation markers | |
| Nuclear antigens (PCNA, Ki67) | |
| Thymidine labeling index | |
| Squamous differentiation markers | |
| Cytokeratins | |
| Transglutaminase type I | |
| Involucrin | |
| Nuclear retinoid receptors | |
| Retinoic acid receptors (RARs) | |
| Retinoid X receptors (RXRs) | |

*General genomic markers*

*Micronuclei*

Micronuclei are chromosome or chromatid fragments formed in proliferating cells during cell division from chromosome nondisjunction resulting from carcinogen-induced DNA damage. They have been studied as markers of clastogen exposure. Their frequency was widely studied in earlier human chemoprevention trials [56–58], and they are still widely studied because they are easy to find and quantify. In the aerodigestive tract epithelium, they form in the proliferating basal cell layer, which gives rise to suprabasal cells that migrate to the epithelial surface, and they can be detected at relatively high frequencies in exfoliated cells of the aerodigestive tract of tobacco-exposed individuals.

A series of pilot trials has shown that micronuclei frequency correlates with target-tissue cancer risk in smokers, who are at high risk for head and neck, lung, esophagus, and bladder carcinomas [57, 58]. In studies of high-risk betel users from India and the Philippines, Stich *et al.* found that both retinol and beta-carotene suppressed micronuclei frequency in more than 90% of the lesions after treatment but that rates of clinical response and suppression of new lesions differed greatly [57, 59, 60]. A large placebo-controlled chemoprevention trial in China revealed significant site-specific suppression of micronuclei (esophageal, not buccal) in subjects receiving retinol, riboflavin, and zinc, but no significant reduction in the number of premalignant esophageal lesions after 1 year of chemopreventive intervention [61]. Data from our group [62, 63] suggest that, over time, retinoids can effectively and steadily suppress micronuclei, although we could not consistently correlate this with clinical and histologic remission [63]. Thus, the presence and frequency of micronuclei seem to quantitatively reflect ongoing DNA damage and genetic instability. However, simple measurements of micronuclei cannot accurately summarize the effects of long-term carcinogen exposure and cumulative effects of genetic change throughout the field.

*Chromosome abnormalities*

The chromosomal and DNA abnormalities seen in

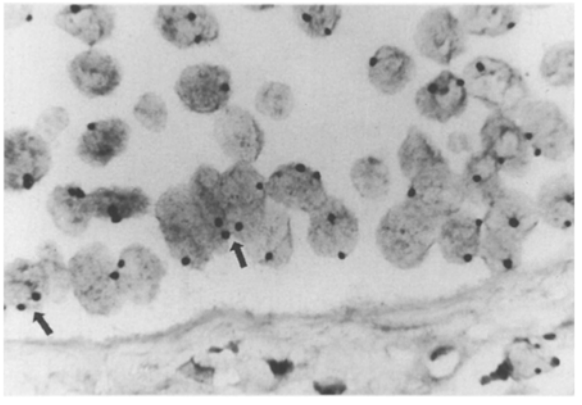

*Fig. 1.* Evidence of chromosome polysomy (i.e., chromosome 9) in dysplastic head and neck epithelium. The arrows are pointing to cells with three chromosome copies indicating genomic instability.

established tumors of the head and neck [64–66] reflect multiple genetic events. Up to now, most studies of these abnormalities have been done in cell lines derived from tumors or subjected to short-term culture. Unfortunately, this method of study subjects the results to the influence of cell culture conditions, particularly through the preferential outgrowth of certain cellular clones and the acquisition of *ex vivo* chromosomal changes [67]. Furthermore, solid-tumor cytogenetic studies have been hampered by the low frequency of mitotic figures from direct preparation, the suboptimal quality of chromosome preparation techniques, and the significant complexity of cytogenetic changes [68].

Nevertheless, short-term cultures have been useful in analyzing complex abnormalities such as rearrangements, translocations, amplifications, and deletions. In fact, several nonrandom chromosomal alterations have been identified in this way: loss on chromosomes 3p, 5q, 8p, 9p, 18q, and 21q [67, 69, 70]; gain or amplification at 3q, 5p, 7p, 8q, and 11q13 [67, 63–71]; and breakpoints at 1p36, 3q21, 5p14, 7p13, 9q32, and 1q13 [71]. However, few karyotypic changes in premalignant tissues of the aerodigestive tract have been identified [72, 73].

This is changing, thanks to ISH and comparative genomic hydribization (CGH). Recently, ISH techniques have allowed the direct visualization of chromosomal abnormalities in interphase cells [74, 75]. They have also been applied to many tumor cell lines and dissected tumor material [76], as well as paraffin-embedded tissue sections, in combination with chromosome-specific DNA probes, and enzyme-mediated immunohistochemical procedures [43, 77–79].

As noted above, we have already used ISH to study head and neck tumor samples containing adjacent normal and premalignant epithelium. In brief, we subjected samples to nonisotopic ISH with specific centromeric DNA probes for chromosomes 7 and 17 to determine (a) the stage of the multistep tumorigenic process at which the specific numerical chromosome changes can be detected and (b) whether histologic progression is accompanied by accumulation of genetic alterations [43]. Polysomy was defined as the presence of 3 or more copies of a chromosome/cell (Fig. 1). We found that polysomy increased from 35% to 90% of the cases as tissue moved from normal epithelium adjacent to tumor through premalignant lesions to squamous cel carcinoma. These results strongly support the multistep tumorigenesis theory and make polysomy a useful genetic biomarker of accumulated genetic damage or instability and ongoing genetic damage. We have subsequently shown that in individuals with leukoplakia or erythroplakia, increased degree of chromosome abnormalities in the lesions have a higher risk of developing oral cancers [80]. Interestingly, half of these tumors occurred away from the initial biopsy site, further proof of field cancerization and that genomic instability in one part of a field increases risk for the rest of the field.

Together, ISH and CGH have revealed much about chromosomal abnormalities as biomarkers. Both techniques yield specific information regarding chromosome amplification and have the advantage of application in primary tumor materials. CGH allows differentially labeled tumor DNA and normal reference DNA to be hybridized simultaneously with normal metaphase spreads. Deletions or amplifications are seen as changes in the ratio of the intensities of the two fluorochromes along the target chromosomes [81]. CGH analysis of 13 head and neck tumors identified deletions in the consensus regions of chromosomes 3p, 5q, and 19, whereas frequently occurring overpresentations were observed in 3q and 5p. Moreover, three tumors had high

levels of amplification that were mapped to 3q26→qter and four demonstrated a relative overpresentation of 11q13, the region that harbors the *PRAD1/cyclin D1* gene [82].

Besides ISH and CGH, new polymerase chain reaction (PCR)-based techniques have allowed specific analysis of DNA from even minute specimens and identification of allelic losses or deletions that mark the inactivation of certain tumor suppressor genes. This is especially useful in studying the inactivation of recessive oncogenes. Such inactivation is thought to be a two-step process frequently involving a point or small mutation in one allele and loss of genetic material through rearrangement or deletion of the other [83]. Restriction fragment length polymorphism (RFLP) and, more recently, microsatellite markers (small highly polymorphic DNA repeated units that occur throughout the human genome) have greatly facilitated allelotyping [84]. By identifying regions of loss of heterozygosity (LOH) at 3p, 5q, 9p, 11q, 13q, and 17p, Ah-See *et al.* [85] and Nawroz *et al.* [86] have comprehensively allelotyped primary head and neck squamous cell carcinoma.

LOH on 3p has been inferred from statistical analyses of RFLP [87, 88], and LOH and deletions of 3p have been implicated in different types of lung cancer [89–91]. Yet, despite the existence of several candidate genes [92], there is still no definitive evidence of a tumor suppressor gene on 3p. Several studies have suggested that this region of loss is more complex because of the possible overlap of three distinct but juxtaposed suppressor regions [93, 94]. Chromosome 5q, near the *APC/MCC* locus, which is involved in colorectal carcinoma, esophageal, and breast cancer [97], contains the most common region of loss in head and neck squamous carcinoma. Though this implies a much broader role for this locus in the development of squamous carcinoma as well as adenocarcinoma, mutation analysis of candidate genes on 5q is needed. The striking finding of LOH on chromosome 9p21–22 in human tumors is consistent with previous reports of a putative suppressor gene near the interferon locus [98]. Furthermore, homozygous deletion of this region has been previously implicated in the progression of many human neoplasms, including brain tumors [99], melanoma [100], bladder cancer [101], and lung cancer [102]. Contained within this region is a cell-cycle gene called *p16 (MTS-1 or CDKN-2)*.

Chromosome 11q is implicated in head and neck squamous cell carcinomas because of the putative involvement of the *cyclin D1* gene, and LOH on 11q may in fact represent amplification [103–105]. LOH on 13q occurs in more than 50% of these tumors and is commonly associated with inactivation of the retinoblastoma gene (*Rb*). However, immunohistochemical analysis has revealed *Rb* inactivation in only a small percentage of tumors with loss of 13q [106], suggesting that further mapping may reveal a second tumor suppressor gene nearby.

Clearly, some tumor suppressor genes have been localized on affected chromosomes, but many others have not. Thus, the fine mapping of allelic losses may lead to the characterization of these unknown tumor suppressor genes. If so, then this will reveal more about the multistep process as well as the biological and clinical behavior of these neoplasms.

## Specific genetic markers

### *Proto-oncogenes*

#### *Ras*

The *ras* genes [H-*ras*, K-*ras*, and N-*ras*] encode 21-kDa guanosine-triphosphate (GTP)-binding proteins ($p21^{ras}$) that attach to the inside of the cell membrane and transduce molecular signals to the nucleus. The p21 proteins have intrinsic GTPase activity that eventually leads to their own inactivation. This inactivation is greatly enhanced by the GTPase-activating protein, which binds to the *ras* gene transductional domain (or effector domain of p21) [107].

Nearly all relevant *ras* mutations occur in codons 12, 13, or 61 [108], and 10–15% of all human cancers harbor such mutations. H-*ras* mutations mainly occur, though at low frequency, in thyroid and bladder cancer [109, 110]. N-*ras* mutations mainly occur in malignancies of the hematopoietic system [108]. K-*ras* mutations are mainly found in adenocarcino-

mas, i.e., pancreatic cancer [111], colorectal cancer [112], and lung adenocarcinomas [113] (their incidence in other histologic types of lung cancer is low [114]). Almost all of these mutations in lung adenocarcinomas occur in smokers and imply a worse prognosis. The majority of mutations at codon 12 K-*ras* is guanine to thymine transversion resulting possibly from the nature of the carcinogens [113].

Few *ras* mutations have been identified in primary head and neck tumors [115–117]. Furthermore, they are rare in oral squamous cell carcinomas in whites. Interestingly, however, *ras* (H-*ras*) mutations were frequent in oral carcinomas in an Indian population of tobacco (quid) chewers. This discrepancy is difficult to explain, assuming the common carcinogen. Perhaps it is due to the longer exposure of mucosa to concentrated levels of carcinogens in chewers versus smokers or to the different strains or species of tobacco used [118]. In addition, the discrepancy in incidence of *ras* mutations between head and neck and lung cancer may be partially due to the predilection of K-*ras* mutations for adenocarcinomas. Kuo *et al.*'s comparative study of $p21^{ras}$ expression in adjacent sections of oral squamous cell carcinomas, epithelial dysplasias, hyperkeratoses, and normal oral mucosae revealed positive staining in 16.7% of mucosae; this progressively increased to 92.2% of squamous cell carcinomas [119]. Furthermore, Kuo *et al.* found that p21 *ras* expression was significantly associated with smoking habits and daily or total betel quid consumption [119].

*Epidermal Growth Factor Receptor (EGFR)*

Epidermal growth factor (EGF) is a potent mitogenic polypeptide that stimulates proliferation of target cells through interaction with its surface receptor, *EGFR*, a 170-kDa transmembrane glycosylated phosphoprotein with tyrosine kinase activity encoded by the *ErbB1* oncogene of the avian erythroblastosis virus [120]. *EGFR* also mediates signal transduction for transforming growth factor-α (TGF-α) [121]. Activation of the *EGFR* kinase results in both autophosphorylation and phosphorylation of tyrosine residues of several proteins *in vitro* [122–124].

Evidence for the role of *EGFR* in tumorigenesis is provided by the observation that, when it or one

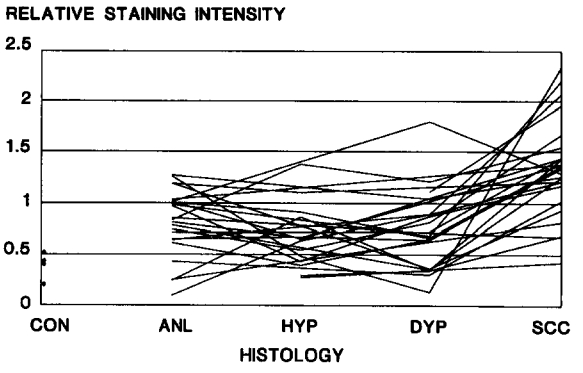


*Fig. 2.* Quantitation of EGFR expression during tumorigenesis. Note that each line represents each sample's relative staining intensity of EGFR expression in different histologies. Overall sample numbers were 4 control epithelia, 28 normal epithelia adjacent to tumor (ANL), 15 hyperplasia (HYP), 24 dysplasia (DYP), and/or 36 squamous cell carcinomas (SCC). Note also that two samples of SCC show lower EGFR expression than their premalignant counterparts (from Shin DM, *et al.*: Cancer Res 54: 3153–3159, 1994).

of its ligands *EGF* or *TGF-α* is overexpressed in transfected cells, it results in malignant transformation [125]. *EGFR* gene amplification and/or overexpression has been found in head and neck squamous cell carcinomas and their cell lines [126–129] some of which also produce TGF-α [127]. In a hamster model of oral carcinogenesis the development of tumors is associated with increased *EGFR* expression [130].

Now, there is even further evidence that *EGFR* overexpression is one of the early events in the multistep process that leads to head and neck carcinomas [131, 132]. In particular, Grands and Tweardy [131] have measured the frequency of increased *TGF-α* and *EGFR* mRNA production in normal-appearing mucosae from head and neck cancer patients versus normal mucosae from controls. They found that *EGFR* expression increased an average 29-fold in most normal-appearing mucosae (91% of samples) of head and neck cancer patients and an average 69-fold in most tumor specimens (92% of the samples) compared with the normal mucosa of nonsmokers. Our own group examined tissue specimens from head and neck cancer patients containing adjacent normal-appearing mucosae and/or premalignant lesions, using anti-*EGFR* monoclonal antibody by immunostaining and computerized

microscopic image analysis [132]. The relative staining intensity to *EGFR* was two fold higher in histologically normal epithelium adjacent to the tumors than in normal control epithelium from nonsmokers. Furthermore, *EGFR* expression increased with histologic progression, particularly, between dysplasia and squamous cell carcinoma, suggesting that upregulation of *EGFR* expression was involved in the multistep process of tumorigenesis (Fig. 2).

Although the abnormal expression of *EGFR* in carcinogen-exposed epithelium may be part of a regenerative process, there is evidence that it coexists with or results from abnormal genotypic changes (i.e., genomic instability). The proof lies in the similar patterns of increased chromosome 7 and 17 polysomy during histologic progression [43]. Therefore, the stepwise increase of *EGFR* expression, in concert with genomic instability, may interfere with normal growth regulation and thus play a role in head and neck tumorigenesis.

PRAD-1 (CCND1)

Cytogenetic analysis of head and neck squamous cell carcinomas has identified frequent abnormalities at 11q13, including rearrangements and the presence of homogeneously staining regions [65]. Interestingly, molecular studies have shown that some genes in this region (*int*-2, *hst*-1, *gst*-π, and *bcl*-1) are amplified in 30–50% of head and neck cancers [103, 104, 133]; thus, these genes may take part in the development and/or progression of these tumors. However, in spite of their amplifications, no mRNA expression of *int*-2, *hst*-1, or *bcl*-1 genes have been consistently demonstrated in fresh head and neck tumors or cell lines [96, 134]. The *int*-2 and *hst*-1 genes encode fibroblast growth factors [135], while *bcl*-1 gene is activated by the t(11;14) translocation in some B-cell lymphomas/leukemias [135].

*PRAD*-1, a gene on 11q13, originally isolated as a gene overexpressed by juxtaposition to the parathyroid hormone gene on 11p15 in parathyroid adenomas [136], lies between the *bcl*-1 gene (telomeric) and *hst*-1 and *int*-2 genes (centromeric) [105]. Together, these four genes form an amplicon implicated in the pathogenesis of different human carcinomas [136, 137]. *PRAD*-1 encodes *cyclin* D1, which is committed to controlling the cell cycle at the $G_1$-S transition by interacting with the *Rb* gene product [139, 140, 141] and by binding and activating cdk-4 and cdk-6 kinases [136, 142]. *pRb* and *cyclin D1* seem to be two components of the same pathway since cells whose *pRb* has been inactivated through mutations or complexing to DNA virus oncoproteins no longer require *cyclin D1* expression to progress through the cell cycle [143, 152]. Cells that overexpress *cyclin D1* proliferate abnormally, have a shorter $G_1$ phase, and depend less on growth factors [140, 144]. Transfection studies have shown that *PRAD-1* may also function as an oncogene in cooperating with other oncogenes in cellular transformation: though it might not help initially transform squamous cells, it may confer some growth advantage to cells already transformed [145].

*PRAD*-1 is amplified and overexpressed in breast and squamous cell carcinomas [105, 134] and amplified in approximately 30–50% of primary head and neck cancers [139, 146]. Its amplification has been correlated with high cytologic grade, infiltrative growth pattern, hypopharyngeal site, and lymph node involvement in recent studies [139, 146, 147]. Its overexpression has been associated with a more rapid and frequent recurrence of head and neck squamous cell carcinomas, resulting in shortened disease-free and overall survival in operable patients [147]. The same study showed a high correlation between amplification and overexpression of the gene, indicating that amplification might be the main mechanism of gene activation in these tumors. However, other studies suggest alternative mechanisms such as translocations, mutations in the gene regulatory regions, or mRNA stabilization [136, 138, 149].

To identify the region amplified, we studied head and neck squamous cell lines and the tissue sections from which they were established using ISH, a cosmid probe for the *int*-2 gene, and a total chromosome 11 painting probe. One line showed no amplification, but three did: on chromosome 11 distal to the single copy gene in two and on another chromosome in the third. *Int*-2 probing of paraffin sections containing adjacent premalignant lesions revealed two cases of amplification in dysplastic lesions and one case of amplification at the hyperplasia-to-dys-

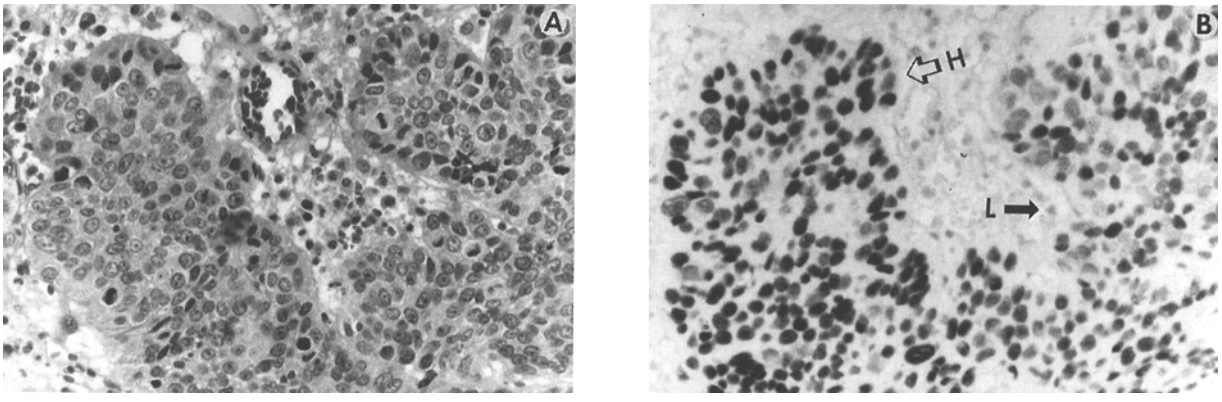


*Fig. 3.* (A) Squamous carcinoma of the head and neck. (B) Heterogeneous expression of p53 protein in the same tumor specimen. (H indicates high p53 and L shows low p53 expression.)

plasia transition. Together, these results suggest that *int*-2 can be amplified in premalignant lesions before tumor development [150]. But the implications for head and neck tumorigenesis need to be further investigated.

*Tumor suppressor genes*

*p53*

The *p53* gene, which maps to the short arm of chromosome 17(17p13), encodes a nuclear protein. This protein induces cell-cycle arrest at the $G_1$ checkpoint, allowing DNA repair or induction of cell apoptosis in response to genotoxic damage. It also functions as a 'guardian of the genome' [151].

Many types of *p53* alterations (rearrangements, deletions, insertions, and point mutations) have been observed in a wide variety of cell lines and tumors, the most frequent being point mutations primarily in exons 5–8. Consequently, *p53* mutation patterns have been analyzed to identify specific changes associated with particular carcinogens [152]. So far, investigators have found frequent *p53* inactivation in head and neck squamous cell carcinomas [153–155], slightly less frequent inactivation in dysplasias [156], and more frequent mutation in smokers and drinkers, versus nonsmokers and nondrinkers [157]. Moreover, CpG mutations (endogenous mutation site) have proved rare in smokers and drinkers, while they constituted all of the mutations found in nonsmokers and nondrinkers. Finally, cigarette smoking has been linked to *p53* mutations at nonendogeneous sites (most commonly GC–TA and CG–AT), thus providing molecular evidence for the well-known epidemiologic association between smoking and head and neck cancer.

Stabilization of the mutant *p53* protein allows its immunohistochemical analysis in tissues, while wild-type *p53* protein is usually undetectable or low (Fig. 3). Immunocytochemistry has detected abnormal *p53* in approximately half of head and neck cancer specimens examined. Interestingly, *p53* is often abnormally expressed in histologically normal epithelium (adjacent to tumors) (25% of cases) more frequently in dysplastic epithelium adjacent to tumors (45%) [158]. This gradual increase of *p53* expression from normal tissue to squamous cell carcinomas supports the hypothesis of multistep tumorigenesis (Fig. 4).

The pathophysiological consequences of losing *p53* function are not well understood. For example, such a loss in sun-exposed skin can lead to the outgrowth of cell clones that resist damage-induced apoptosis [159]. This means that genetically altered cells accumulate more rapidly with continued exposure to carcinogens and explains the recent finding that *p53*-positive premalignant lesions adjacent to tumors have higher levels of chromosome polysomies than *p53*-negative lesions [160].

Two recent studies of *p53* mutation strongly argue for a multifocal, polyclonal process of tumorigenesis. One found frequent *p53* mutations in initial head and neck cancers and second primary tumors

of the aerodigestive tract [161], in all cases, the mutation sites differed between initial and second primary tumors, strongly suggesting a different clonal origin for the initial and second primary tumors. This confirmed an earlier study finding of distinct, multifocal cells expressing aberrant *p53* in distal epithelia of head and neck cancer patients [162].

Obviously, *p53* mutations are promising biomarkers. To determine how *p53* alterations in at-risk tissue might affect clinical outcome, we prospectively assessed cancer-free patients with premalignant oral lesions for *p53* protein accumulation and evaluated the response to 13-*cis* retinoic acid (13cRA) [163, 164]. Retinoids can apparently modulate *p53* mRNA and protein levels associated with carcinogenesis in certain *in vitro* systems [165]. We found that 89% of premalignant lesions expressed *p53*, while 8 controls from healthy nonsmokers did not. Levels of *p53* protein accumulation increased with histological progression. A significant correlation between levels of *p53* protein accumulation and lesion resistance to 13cRA therapy was found. This finding is possibly explained by the fact that cells that have lost wild-type *p53* function may not undergo apoptosis with 13-cRA therapy. However, there was no modulation of *p53* protein expression by 13-cRA therapy. *P53* alterations may be a valuable marker for indentification of individuals at high risk of developing resistance in chemoprevention. We also found that patients with *p53*-positive tumors had shorter survival than those with *p53*-negative tumors, mainly due to the earlier and more frequent development of second primary tumors [166]. Additionally, *p53* alterations may help possibly target patients for gene therapy [167].

*p53* and *cyclin D1* represent mechanistically independent growth regulatory systems in head and neck cancer, which when altered, both abrogate $G_1$ regulatory events [168]. The existence of a subset $G_1$ of tumors with alteration of both genes supports the notion that concomitant inactivation of both regulatory pathway selectively enhances tumor growth [169]. The *cyclin D1/pRb* pathway includes at least two tumor suppressor genes (*pRb* and *p16/CDKN2*) and at least one proto-oncogene (*cyclin D1*) and may turn out to be as important as the *p53/p21* pathway in carcinogenesis. Ongoing research is addressing the role of these complex genetic events that might complement the effects of *p53* providing the ultimate selective growth advantage to cells.

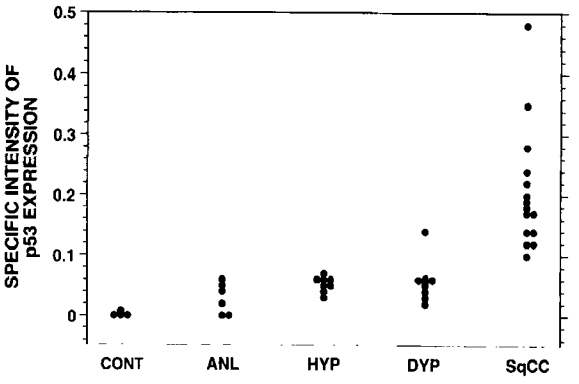


*Fig. 4.* Quantitation of p53 expression in positively p53 expressed samples during head and neck tumorigenesis. Note that the normal epithelium adjacent to tumor (ANL) showed higher p53 expression than normal control epithelium (CONT) ($P = 0.053$) and increased further to hyperplasia (HYP) ($P = 0.038$) and dysplasia (DYP). From dysplasia to squamous cell carcinoma (SqCC), p53 increased dramatically ($P < 0.001$) (from Shin DM, *et al.*: Cancer Res 54: 321–326, 1994).

### $p21^{WAF1/CIP1}$

The ability of *p53* to activate transcription of specific sequences suggests that the genes it induces may mediate its biological role as tumor suppressor. One such highly induced gene is $p21^{WAF1/CIP1}$ (so named for the simultaneously but separately cloned wild-type *p53*-activated fragment-1 [WAF1] and cdk-interacting protein-1 (CIP-1]) [170, 171]. $p21^{WAF1/CIP1}$ is directly regulated by *p53* and can itself suppress tumor cell growth in culture by inhibiting cyclin-dependent kinases and inducing $G_1$ arrest or apoptosis. However, expression of the gene product can also occur independently of *p53*, such as during the Myo-dependent formation of myotubes from myoblasts [172–174]. *P53* independent induction of $p21^{WAF1/CIP1}$ occurred also in breast carcinoma and prostatic carcinoma cell lines treated with lovastatin [175] and in human prostate carcinoma cell lines treated with all-*trans* retinoic acid (aTRA) and 9cRA [176] among others. Transfected $p21^{WAF1/CIP1}$ inhibits growth of both human tumor cell lines [170] and normal diploid fibroblasts [171]. *In vitro*, $p21^{WAF1/CIP1}$ complexed with the proliferat-

ing call nuclear antigen (PCNA) can inhibit DNA replication [177].

Mutations within the coding portion of the *WAF1* gene were undetectable in a large series of human tumors, suggesting that *WAF1* mutations *per se* may not play an important role in the onset or progression of these malignancies [178]. The expression of $p21^{WAF1/CIP1}$ in adult human tissues has been examined in a variety of tissues [179]. In colonic epithelium there was a topological correlation in $p21^{WAF1/CIP1}$ immunostaining, with staining observed in the upper half of the colonic crypts, located thus $p21^{WAF1/CIP1}$ expression in the post-proliferative compartment [180, 181]. The same team of investigators extended their observations with study of $p21^{WAF1/CIP1}$ expression in colorectal adenomas and carcinomas, and found a global decrease and loss of the distinct compartmentalization of this protein. The results suggest that $p21^{WAF1/CIP1}$ alterations seem to be a relatively early event in the neoplastic process in this tumor. In head and neck squamous carcinomas there has been little evidence for implication of $p21^{WAF1/CIP1}$ in the tumorigenesis process, but research in relation to other relevant genes in this tumor is currently ongoing in our laboratory and others.

*MTS1*

Within the 9p21 region lies a putative tumor suppressor gene, *p16/MTS1/CDKN2*, that encodes a cell-cycle regulator protein p16. This protein negatively controls cell proliferation by binding to cdk4 and thus inhibiting the interaction of the cyclin D-cdk4 complex with *pRb* [182, 183]. This links *cyclin* D-cdk4 and pRb in a common pathway, where loss of p16 may lead to uncontrolled cell growth and accelerate the carcinogenic process.

Interestingly, several recent studies have demonstrated homozygous deletion of the 9p21 region in a variety of neoplasms including leukemia, bladder cancer, glioma, melanoma, and lung cancer [100, 101, 184]. Allelic losses at the 9p21–22 locus also occur frequently in head an neck squamous cell carcinomas and just as frequently in early preinvasive lesions (including dysplasia and carcinoma *in situ*) [185]. This provides strong molecular evidence for the early inactivation of a putative tumor suppressor gene on 9p leading to initiation or early progression of head and neck cancers. Sequence analysis of the *p16* gene in primary head and neck cancers that have lost chromosome 9p has revealed very rare point mutations [186–188]. This suggests that some mutations might escape detection, an alternative mechanism for inactivation of this gene, or that a second suppressor locus resides within the critical region of 9p21 in head and neck cancers. Several factors may contribute to the apparent discrepancy between homozygous deletions and loss of expression of the p16 gene [188]. First, *MTS1* may be a codominant tumor suppressor, with loss of one copy contributing to deregulated cell proliferation. Second, hypermethylation of a 5′ CpG island of p16 may transcriptionally silence the full-length p16, as seen in lung and head and neck cancers, glioma cell lines, and fresh tumors without deletions or mutations [189]. The potential mechanism(s) for inactivation of the gene function as well as the exact role of the gene in the multistep head and neck tumorigenesis process need to be further studied.

*Proliferation markers*

Proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase δ, plays a critical role in the initiation of cell proliferation [190, 191]. It is one of the cell-cycle-regulated proteins in the DNA replication complex whose expression is associated with the late $G_1$-S and early $G_2$ phases [192]. Since proliferation is thought to be an early marker of disordered cell growth, it has been hypothesized that proliferation increases as premalignant lesions become more advanced and that the distribution of proliferating cells in tissue may reveal the regulatory mechanisms that function abnormally during the multistep process.

PCNA seems to be a useful biomarker in the multistep tumorigenesis process. Using immunohistochemical staining we assessed the proliferative changes during tumor development in a series of head and neck cancer samples containing the whole spectrum of premalignant lesions [193]. Our chief finding was a fourfold increase in PCNA expression in the basal layer, extending to the suprabasal layer

in normal epithelium adjacent to cancers, versus low expression confined to the basal layer in the normal control epithelium of nonsmokers (Table 2). PCNA expression and thus dysregulation of proliferation increased as tissue progressed from adjacent normal epithelium to squamous carcinoma. Huang *et al.* studied PCNA expression in 169 oral epithelial lesions, including carcinoma *in situ*, dysplasia, epithelial atypia and hyperplasia [194]. They found a significant predilection for basal or suprabasal staining in premalignant or malignant lesions versus strictly basal staining in normal epithelium. Other studies by Gorgoulis *et al.* and Girod *et al.* have found a positive correlation between immunohistochemical *p53* expression and PCNA expression in premalignant and malignant lesions [195, 196].

### *Squamous cell differentiation markers*

Unlike skin epithelium, the aerodigestive tract does not undergo squamous differentiation under normal physiologic conditions. However, it does differentiate along the squamous pathway during carcinogenesis [197]. In fact, over 90% of oral cavity tumors are squamous cell carcinomas [5]. Consequently, some preclinical and clinical studies have focused on markers of differentiation in the oral cavity, especially cytokeratins, involucrin, and transglutaminase I.

#### *Cytokeratins*

The cytokeratins, a family of at least 19 intermediate-size filaments, are good squamous differentiation markers because (a) they are expressed in different combinations in various human epithelial tissues and (b) their pattern of expression correlates with distinct types of epithelial differentiation [198]. Their expression patterns are greatly altered *in vitro* and *in vivo* during vitamin A deficiency, cigarette-smoke exposure, and carcinogenesis [199, 200]. The most useful markers seem to be keratin 1 (K1) and keratin 19 (K19): K1 because it marks carcinogenic changes in oral epithelia except in the gingiva, where it occurs normally, and K19 because its expression is increased and extends to suprabasal layers of epithelium in premalignant and malignant lesions [201].

Gimenez-Conti *et al.*, in studies with a monospecific antibody for K1, found that K1 expression increased during 7,12-dimethyl-benz(a)anthracene (DMBA)-induced oral carcinogenesis in the hamster cheek pouch *in vivo* [202]. The normal hamster cheek pouch epithelium, like human buccal mucosa, does not express K1, whose expression seems to be limited to hyperplastic cells in this model. Copper *et al.* [203], attempting to define markers of subsequent invasive squamous cell carcinoma, investigated different cytokeratin profiles using monoclonal antibodies to cytokeratins 8, 10, 13, and 19 and other monoclonal antibodies designated K931, K984, E46, Ki67, and UMA9. (Except for Ki67 all these monoclonal antibodies recognize epithelial differentiation antigens.) They found that K19 expression increased more than 3-fold in normal-appearing exfoliated mucosa versus controls. K19 is normally present in small amounts in the basal layer

*Table 2.* Labeling index of PCNA expression

| Histology* | Labeling index, mean ± SE** | | | |
|---|---|---|---|---|
| | Basal layer | Parabasal layer | Superficial layer | Overall |
| NC (N = 6) | 0.06 ± 0.01 | 0.07 ± 0.02 | 0 | 0.04 ± 0.01 |
| ANL (N = 25) | 0.11 ± 0.01 | 0.22 ± 0.02 | 0.06 ± 0.01 | 0.13 ± 0.01 |
| HP (N = 13) | 0.29 ± 0.04 | 0.54 ± 0.04 | 0.12 ± 0.02 | 0.32 ± 0.03 |
| DP (N = 22) | 0.55 ± 0.04 | 0.68 ± 0.03 | 0.22 ± 0.02 | 0.48 ± 0.03 |
| SCC (N = 33) | NA | NA | NA | 0.76 ± 0.02 |

* NC = normal control; ANL = normal epithelium adjacent to tumor cells; HP = hyperplasia; DP = dysplasia; SCC = squamous cell carcinoma.

** NA = not applicable. (From Shin DM: J Natl Cancer Inst 85: 971–798, 1993.)

of noncornifying regions of oral epithelia, but in premalignant lesions as well as squamous cell carcinomas, its expression is increased and extends to suprabasal layers [201]. Cooper's study extends this observation to normal-appearing 'condemned' mucosa. The high level of K19 expression might reflect a failure of maturation since there is an inverse relationship between the cellular levels of the terminal differentiation marker, involucrin [201], and K19.

*Involucrin and transglutaminase I*

Involucrin is produced during squamous differentiation in epidermis *in vivo* and in cultured keratinocytes and tracheal cells. Because it cross-links extensively with the membrane-associated enzyme type I transglutaminase, involucrin and transglutaminase I levels both increase during squamous differentiation [204, 205]. As immunohistochemical staining has shown, involucrin is expressed in the superficial layer of normal and hyperplastic oral and laryngeal epithelia, but throughout the thickness of the epithelium (including the basal and suprabasal layers) in dysplasias and squamous cell carcinomas in irregular and focal fashion [206, 207]. Furthermore, while involucrin staining is consistently strong in human premalignant oral lesions *in vitro*, immunoreactivity for transglutaminase I inversely correlates with the degree of dysplasia suggesting uncoupling of the keratinocyte programming [207].

Because aerodigestive tract epithelia differentiate along the squamous pathway in carcinogenesis and because retinoids inhibit this abnormal differentiation *in vitro* [207, 208], the effect of retinoids on the markers K1, involucrin, and transglutaminase I has been studied. Lippman *et al.* studied the effect of retinoids on marker expression and modulation as part of a two-phase trial of 13-cRA treatment in these oral leukoplakia [209]. They found that the pattern of staining was homogeneous in suprabasal layers at early stages (hyperplasia) but heterogeneous with basal layer extension at later stages (dysplasia) of carcinogenesis. Early results suggest that 13cRA may suppress the abnormal expression of squamous markers in as many as one third of lesions [209].

*Genetic susceptibility markers*

Since only a fraction of individuals exposed to tobacco develops smoking-related cancers, genetic predisposition might play an important role in tumor formation [210]. Indeed, as Sellers *et al.* and Harris have shown, almost every phase of the multistage process of carcinogenesis can be modified by genetically determined or acquired differences in modulation of carcinogenic exposures [210, 211]. One of the factors that determine an individual's risk is how their cells interact with carcinogens (e.g., activate procarcinogens), including how they repair carcinogen-induced DNA damage and how fast these cells accumulate genetic abnormalities.

Cytogenetic assays in peripheral blood lymphocytes have been extensively used to survey exposure and response of humans to genotoxic agents because it is thought that genetic changes observed in lymphocytes reflect critical events in the carcinogenesis in the affected tissues [212]. One such assay measures the frequency and number of *in vitro* bleomycin-induced breaks to gauge mutagen sensitivity [213]. As demonstrated in two case-control studies by Spitz *et al.*, *in vitro* bleomycin-induced sensitivity (defined as $> 0.8$ chromosome breaks/cell) and hypersensitivity ($> 1.0$ breaks/cell) are independent risk factors for head and neck cancers, after adjustment for tobacco and alcohol use [214, 215]. The second study also found that mutagen sensitivity entails a smoking-adjusted odds ratio of 2.9 (95% confidence limits 1.5–5.4) [215]. Further analysis suggests that (a) cigarette smoking combined with mutagen sensitivity multiplies effect and (b) individuals prone to develop second primaries have the highest degrees of mutagen sensitivity [216, 217]. Interestingly, bleomycin-induced breaks are not randomly distributed in patients with head and neck cancer but occur most often in chromosomes 3 (3p21, 3q21) and 7 (7q22, 7q32), chromosomal sites thought to harbor genes important for tumor development [218]. These same allelic losses have been detected in different regions of the tumor field, raising the possibility that inherent sensitivity also involves the predisposition of relevant genetic loci to instability. A multicenter phase III randomized chemoprevention trial to prevent SPTs involving

more than 1000 patients is ongoing through M.D. Anderson Cancer Center and its affiliated Community Clinical Oncology Group (CCOG) and the Radiation Therapy Oncology Group (RTOG). This study should establish the definite role of mutagen sensitivity and other biomarkers studied in this setting.

Eventually, many biomarkers used in combination will be needed to predict the risk of tumor development. These include genes involved directly in tumorigenesis (oncogenes and tumor suppressor genes), susceptibility genes that control DNA repair and, phase I and II metabolic enzyme activity (CYP2D6, CYP1A1, GSTM1, epoxide hydrolase, N-acetyl-transferase), and genes that influence rates of cellular proliferation [219].

*Retinoid mechanisms in chemoprevention*

Retinoids are by far the most studied and most active agents in head and neck cancer chemoprevention [7, 21, 24, 26, 220]. Vast data from epidemiologic, *in vitro*, animal, and human studies indicate that these agents are effective inhibitors of early stages of carcinogenesis in a number of target tissues [7, 220–222]. Clinical retinoid trials show that retinoids have significant chemopreventive activity in the head and neck, lung, bladder, skin, cervix, and ovary [7, 12, 21, 222–225], presumably due to their known regulatory effects on cell differentiation, cell proliferation, and apoptosis [226, 227]. Retinoids are physiologic regulators of tissue differentiation and restore regulation of growth and differentiation in certain premalignant and malignant cells *in vitro* and *in vivo* [226]. However, in cultured keratinocytes and squamous cell carcinomas, retinoids inhibit abnormal squamous differentiation [228] and reverse keratinization by restoring the ability to respond to normal growth control mechanisms. Retinoids enhance cellular interactions by restoring anchorage dependence, enhancing gap junctional communication, and inducing of integrin expression [227]. This enhancement of the interactions between cells may be important for expression of tissue-specific genes and may block the tumorigenesis pathway.

Their ability to regulate gene expression and intervene in signal transduction is the most plausible mechanism by which retinoids can modulate differentiation and growth of malignant cells and suppress progression of premalignant lesions. Such modulation has been seen in proto-oncogenes, tumor suppressor genes, and cell-regulatory kinases [227], as well as in many cell-surface receptors for cytokines and growth factors, with the sum effect of subverting growth-stimulatory signals mediated by different receptors. They can additionally intervene in intracellular signaling via second messengers [227]. To regulate gene expression, they transmit signals to the cell nucleus. To do so, they bind an activate specific nuclear receptors that then bind to DNA.

The nuclear retinoid receptors play a major role as ligand-activated DNA-binding proteins in a series of events that culminates in the modulation of nuclear gene transcription by intraction with response elements in the promoter region of specific genes. Two major types of retinoid nuclear receptors have been identified: RARs and RXRs. Each in turn has at least three subtypes: $\alpha$, $\beta$, $\gamma$. The ongoing study of nuclear retinoid receptors has revealed clear distinctions among the classes and subclasses of receptors in respect to their biologic functions, ligand affinities, and tissue distribution [229]. It seems that the two receptor classes represent distinctly different retinoid response pathways. The involvement of RAR-$\alpha$ in acute promyelocytic leukemia [230], RAR-$\gamma$ in teratocarcinoma [231], and RAR-$\beta$ in oral carcinogenesis [232] suggests a critical role for nuclear retinoid receptors in mediating retinoid activity in human neoplastic processes. The next step is to assume that abrogation of their function or expression results in cancer development.

Of the retinoid nuclear receptors, RAR-$\beta$ seems to play a major role in aerodigestive tract carcinogenesis [233–235]. One clue is its notable absence in a considerable number of head and neck carcinomas and lung carcinoma cell lines [235, 236]. Another clue is the ability of transfected RAR-$\beta$ to suppress the tumorigenicity of a human lung cancer cell line, thus supporting the hypothesis that links its reduction to cancer development [234]. A third clue is the progressive and selective loss of RAR-$\beta$ expression

at the mRNA level associated with head and neck and lung carcinogenic progression [235, 237]. Finally, a fourth clue is the absence of RAR-β expression in approximately two thirds of oral premalignant lesions, its significant upregulation by isotretinoin treatment from 40% to 90% of the cases with correlation between increased RAR-β expression and clinical response [237]. Thus, RAR-β seems to be the best indicator to date of retinoid chemopreventive efficacy in humans and thus an excellent biomarker candidate.

The biomarkers presented here are all associated with molecular genetic events and the phenotypic changes that characterize field cancerization and multistep tumorigenesis in head and neck cancers. However, before they can be used as definitive risk predictors and intermediate and possibly final endpoints for trials in the near future, they must first be validated through large phase III randomized prevention trials.

## Conclusion

There is strong cellular and molecular evidence that head and neck tumorigenesis involves field cancerization and a multistep process. The future success of cancer chemoprevention will require studies that assess risk and define biomarkers as intermediate end points. Biomarker studies may then be used to help select patients, evaluate the biologic predictive capabilities of biomarkers, and develop novel chemopreventive strategies. At present, important clinical chemoprevention studies are ongoing in conjunction with biomarker studies in the United States and Europe. When these studies are done, they should clarify the biological role of chemopreventive therapy and shed new light on cancer prevention and control.

## Acknowledgement

We would like to thank Ms. Susan Cweren and Ms. Trupti Shah for their technical assistance, to Ms. Karen Shanklin for her preparation of manuscript, and to Jude Richard for his editorial comments. This work was supported in part by NIH grant CA-52051.

## References

1. Boring CC, Squires TS, Tong T: Cancer statistics 1994. CA Cancer 44: 7–26, 1994
2. Parkin DM, Laara E, Muir CS: Estimates of the worldwide frequency of sixteen major cancers in 1980. Int J Cancer 41: 184–197, 1988
3. DeVesa SS, Blot WJ, Stone BJ, Miller BA, Tarone RE, Fraumeni JF: Recent cancer trends in the United States. J Natl Cancer Inst 87: 175–182, 1995
4. Vokes EE, Weichselbaum RR, Lippman SM, Hong WK: Head and neck cancer. N Engl J Med 328: 184–193, 1993
5. Wolf G, Lippman SM, Laramore G, Hong WK: Head and neck cancer. In: Holland JF, Frei E, Bast RC, Kufe DW, Morton DL, Weichselbaum R (eds) Cancer Medicine, 3rd ed. Lea & Febiger, 1993, pp 1211–1275
6. Sporn MB: Carcinogenesis and cancer-different perspectives on the same disease. Cancer Res 51: 6215–6218, 1991
7. Lippman SM, Benner SE, Hong WK: Cancer chemoprevention. J Clin Oncol 12: 851–873, 1994
8. Boone C, Kelloff GJ, Malone WE: Identification of candidate cancer chemoprevention agents and their evaluation in animal models and human clinical trials: A review. Cancer Res 50: 2–9, 1990
9. Meyskens FL Jr: Coming of age: The chemoprevention of cancer. N Engl J Med 323: 825–827, 1990
10. Weber BL: Susceptibility genes for breast cancer. N Engl J Med 331: 1523–1524, 1994
11. Early Breast Cancer Trialists' Collaborative Group: Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. Lancet 339: 1–15, 71–84, 1992
12. Hong WK, Lippman SM, Wolf GT: Recent advances in head and neck cancer: Larynx preservation and cancer chemoprevention. Cancer Res 53: 5113–5120, 1994
13. Heyne K, Lippman SM, Lee JS, Hong WK: The incidence of second primary tumors in long term survivors of small cell lung cancer. J Clin Oncol 10: 1519–1524, 1992
14. Lippman SM, Hong WK: Not yet standard: Retinoids versus second primary tumors. J Clin Oncol 11: 1204–1207, 1993
15. Benner SE, Lippman SM, Hong WK: Chemoprevention of second primary tumors: A model for intervention trials. Eur J Cancer 30A: 727–729, 1994
16. Schatzkin A, Freedman LS, Schiffman MN, Dawsey SM: Validation of intermediate endpoints in cancer research. J Natl Cancer Inst 82: 1746–1752, 1990
17. Zelen M: Are primary cancer prevention trials feasible? J Natl Cancer Inst 80: 1442–1444, 1988
18. Stockman MS, Gupta PK, Pressman NJ, Mulshine JL: Considerations in bringing a cancer biomarker to clinical application. Cancer Res 52 (suppl): 2711–2718, 1992

19. Lippman SM, Spitz MR, Huber MH, Hong WK: Strategies for chemoprevention study of premalignancy and second primary tumors in the head and neck. Curr Opin Oncol 7: 234–241, 1995
20. Lippman SM, Lee JS, Lotan R, Hittelman W, Wargovich MJ, Hong WK: Biomarkers as intermediate end points in chemoprevention trials. J Natl Cancer Inst 82: 555–560, 1990
21. Lippman SM, Benner SE, Hong WK: The chemoprevention of cancer. In: Greenwald P, Kramer BS, Weed DL (eds) Cancer Prevention and Control. Marcel Dekker Inc, New York, 1995, pp 329–352
22. Sharma S, Stutzman JD, Kellof GJ, Steele VE: Screening of potential chemopreventive agents using biochemical markers of carcinogenesis. Cancer Res 54: 5848–5855, 1994
23. Shin DM, Hittelman WN, Hong WK: Biomarkers in upper aerodigestive tract tumorigenesis. Cancer Epidemiol Biomarkers Prev 3: 697–709, 1994
24. Hong WK, Lippman SM, Hittelman WN, Lotan R: Retinoid chemoprevention of aerodigestive cancer: From basic research to the clinic. Clin Cancer Res 1: 677–686, 1995
25. Slaughter DP, Southwick HW, Smejkal W: Field cancerization in oral stratified squamous epithelium: Clinical implications of multicentric origin. Cancer 6: 963–968, 1953
26. Lippman SM, Hong WK: Retinoid chemoprevention of upper aerodigestive tract carcinogenesis. In: De Vita VT, Hellman S, Rosenberg SA (eds) Important Advances in Oncology. JB Lippincot Co, Philadelphia, 1992, pp 93–109
27. Cooper JS, Pajak TF, Rubin P, Tupchong L, Brady LW, Leibel SA, Laramore GE, Marcial VA, Davis LW, Cox JD: Second malignancies in patients who have head and neck cancers: Incidence, effect on survival and implications for chemoprevention based on the RTOG experience. Int J Radiat Oncol Biol Phys 17: 449–456, 1989
28. De Vries N, Snow GB: Prevention of second primary cancers in head and neck patients: New perspectives. Am J Otolaryngol 9: 151–154, 1988
29. Lippman SM, Hong WK: Second malignant tumors in head and neck squamous cell carcinoma: The overshadowing threat for patients with early stage disease (Editorial). Int J Radiat Oncol Biol Phys 17: 691–694, 1989
30. Johnson BE, Linnoila RI, Williams JP, Venzon DJ, Okunieff P, Anderson GB, Richardson GE: Risk of second aerodigestive cancers increase in patients who survive free of small cell lung cancer for more than two years. J Clin Oncol 13: 101–111, 1995
31. Tepperman BS, Fitzpatrick PJ: Second respiratory and upper digestive tract cancers after oral cancer. Lancet 2: 547–549, 1981
32. McDonald S, Haie C, Rubin R, Nelson D, Divers LD: Second malignant tumors in patients with laryngeal carcinoma: Diagnosis, treatment and prevention. Int J Radiat Oncol Biol Phys 17: 457–465, 1989
33. Licciardello JT, Spitz MR, Hong WK: Multiple primary cancer in patients with cancer of the head and neck: Second cancer of the head and neck, esophagus and lung. Int J Radiat Oncol Biol Phys 17: 467–476, 1989
34. Vikram B: Changing patterns of failure in advanced head and neck cancer. Arch Otolaryngol 110: 564–565, 1984
35. Auerbach O, Stout AP, Hammond EC, Garfinkle L: Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer. N Engl J Med 265: 253–267, 1961
36. Aldaz CM, Conti CJ, Klein-Szanto AJP, Slaga TJ: Progressive dysplasia and aneuploidy are hallmarks of mouse skin papillomas: Relevance to malignancy. Proc Natl Acad Sci USA 84: 2029–2032, 1987
37. Konishi F, Morson BC: Pathology of colorectal carcinomas: A colonoscopic survey. J Clin Pathol 35: 830–841, 1982
38. Boone C, Kelloff GJ, Steele VE: Natural history of intraepithelial neoplasia in humans with implications for cancer chemoprevention strategy. Cancer Res 52: 1651–1659, 1992
39. Sozzi G, Miozzo M, Donghi R, Pilotti S, Cariani CT, Pastorino V, Della Porta G, Pierotti MA: Deletions of 17p and p53 mutations in preneoplastic lesions of the lung. Cancer Res 52: 6079–6082, 1992
40. Lee JS, Pathak S, Hopwood V, Tomasovic B, Mullins JD, Baker FL, Spitzer G, Neidhart JA: Involvement of chromosome 7 in primary lung cancer and nonmalignant normal lung tissue. Cancer Res 47: 6349–6352, 1987
41. Hittelman WN, Lee JS, Cheong N, Shin DM, Hong WK: The chromosome view of 'field cancerization' and multistep tumorigenesis. In: Pastorino U, Hong WK (eds) Implication for Chemoprevention Approaches in Chemoprevention Cancer. Thieme Verlag, Stuttgart, 1991, pp 41–47
42. Kim SY, Lee JS, Ro JY, Gay M, Hong WK, Hittelman WN: Interphase cytogenetics in paraffin sections of lung tumors by non-isotopic *in situ* hybridization: Mapping genotype/phenotype heterogeneity. Am J Pathol 142: 307–317, 1993
43. Voravud N, Shin DM, Ro JY, Lee JS, Hong WK, Hittelman WN: Increased polysomies of chromosomes 7 and 17 during head and neck multistage tumorigenesis. Cancer Res 53: 2874–2883, 1993
44. Sundaresan V, Ganly P, Hasleton P, Rudd R, Sinhha G, Blechen NM, Rabbits P: *p53* and chromosome 3 abnormalities, characteristic of malignant lung tumors, are detectable in preinvasive lesions of the bronchus. Oncogene 7: 1989–1997, 1992
45. Hung J, Kishimoto Y, Sugio K, Virmani A, McIntire DD, Minna JD, Gazdar AF: Allele-specific chromosome 3p deletions occur at an early stage in the pathogenesis of lung carcinoma. JAMA 273: 558–563, 1995
46. Kishimoto Y, Sugio K, Hung JY, Virmani AK, McIntire DD, Minna JD, Gazdar A: Allele-specific loss in the chromosome 9p loci in preneoplastic lesion accompanying non-small cell lung cancer. J Natl Cancer Inst 87: 1224–1229, 1995
47. Sugio K, Kishimoto Y, Virmani A, Hung JY, Gazdar AF: K-*ras* mutations are a relatively late event in the pathogenesis of lung carcinomas. Cancer Res 54: 5811–5915, 1994
48. Chung KY, Muchopadhyay T, Casson A, Ro JY, Goepfert

H, Hong WK, Roth JA: Discordant *p53* mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. Cancer Res 53: 1676–1683, 1993
49. Farber E: The multistep nature of cancer development. Cancer Res 44: 4217–4223, 1984
50. Vogelstein B, Fearon ER, Hamilton SR: Genetic alterations during colorectal tumor development. J Engl J Med 319: 525–532, 1988
51. Bouquot JE, Weiland LH, Kurland LT: Leukoplakia and carcinoma *in situ* synchronously associated with invasive oral/oropharyngeal carcinoma in Rochester, Minn., 1935–1984. Oral Surg Oral Med Oral Pathol 65: 199–207, 1985
52. Einhorn J, Wersall J: Incidence of oral carcinoma in patients with leukoplakia of the oral mucosa. Cancer 20: 2189–2193, 1967
53. Mashberg AL: Erythroplasia vs. leukoplakia in the diagnosis of ealry asymptomatic oral squamous carcinoma. N Engl J Med 297: 109–110, 1977
54. Sklar G: Oral leukoplakia. N Engl J Med 315: 1544–1546, 1986
55. Silverman S Jr, Gorsky M, Lozada F: Oral leukoplakia and malignant transformation: A follow-up study of 257 patients. Cancer 53: 563–568, 1984
56. Stich HF: Micronucleated exfoliated cells as indicators for genotoxic damage and as markers in chemoprevention trials. J Nutr Growth Cancer 4: 9–18, 1987
57. Rosin MP, Dunn BP, Stich HF: Use of intermediate endpoints in quantitating the response of precancerous lesions to chemopreventive agents. Can J Physiol Pharmacol 65: 483–487, 1987
58. Lippman SM, Peters E, Wargovich M: Bronchial micronuclei as a marker of an 'early' stage of carcinogenesis in human tracheobronchial epithelium. Int J Cancer 45: 811–815, 1990
59. Stich HF, Rosin MP, Vallejera MO: Reduction with vitamin A and beta-carotene administration of the proportion of micronucleated buccal mucosal cells in Asian betel nut and tobacco chewers. Lancet 1: 1204–1206, 1984
60. Stich HF, Rosin MP, Hornby AP: Remission of oral leukoplakias and micronuclei in tobacco/betel quid chewers treated with beta-carotene and beta-carotene and vitamin A. Int J Cancer 42: 195–199, 1988
61. Munoz N, Hayashi M, Bang LJ, Wahrendorf J, Massimo C, Bosch FX: Effect of riboflavin, retinol, and zinc on micronuclei of buccal mucosa and of esophagus: A randomized double-blind intervention study in China. J Natl Cancer Inst 79: 687–691, 1987
62. Benner SE, Lippman SM, Wargovich MJ, Velasco M, Peters EJ, Morice RC, Hong WK: Micronuclei in bronchial biopsy specimens from heavy smokers: Characterization of an intermediate marker of lung carcinogenesis. Int J Cancer 52: 44–47, 1992
63. Benner SE, Lippman SM, Wargovich MJ, Lee JJ, Velasco M, Martin JW, Toth BB, Hong WK: Micronuclei, a biomarker for chemoprevention trials: Results of a randomized study in oral premalignancy. Int J Cancer 59: 457–459, 1994
64. Mitelman F: Catalog of chromosome aberrations in cancer, 3rd ed. Alan R. Liss, New York, 1988
65. Jin Y, Higashi K, Mandahl N, Heim S, Wennerberg J, Biorklund A, Dictor M, Mitelman F: Frequent rearrangement of chromosomal bands 1p22 and 11q13 in squamous cell carcinoma of the head and neck. Genes Chromosom Cancer 2: 198–204, 1990
66. Osella P, Carlson A, Wyandt H, Milungky A: Cytogenetic studies of eight squamous cell carcinomas of the head and neck. Cancer Genet Cytogenet 59: 73–78, 1992
67. Jin Y, Mertens F, Mandahl N, Heim S, Olegard C, Wennerberg J, Biorklund A, Mitelman F: Chromosome abnormalities in eighty-three head and neck squamous cell carcinomas: Influence of culture conditions on karyotypic pattern. Cancer Res 53: 2140–2146, 1993
68. Tessier JR: The chromosomal analysis of human solid tumors: A triple challenge. Cancer Genet Cytogenet 37: 103–125, 1989
69. Carey TE, Van Dyke DL, Worsham MJ: Nonrandom chromosome aberrations and clonal populations in head and neck cancer. Anticancer Res 13: 2561–2568, 1993
70. Van Dyke DL, Worsham MJ, Benninger MS, Krause CJ, Baker SR, Wolf GT, Drumheller T, Tilley BC, Carey TE: Recurrent cytogenetic abnormalities in squamous cell carcinomas of the head and neck region. Genes Chromosom Cancer 9: 192–206, 1994
71. Cowan JM, Beckett MA, Weichselbaum RR: Chromosome changes characterizing *in vitro* response to radiation in human squamous cell carcinoma lines. Cancer Res 53: 5542–5547, 1993
72. Lee JS, Pathak S, Hopwood V, Tomasovic B, Mullins TD, Baker FL, Spitzer G, Neidhart JA: Involvement of chromosome 7 in primary lung tumor and nonmalignant normal lung tissue. Cancer Res 47: 6349–6352, 1987
73. Mertens F, Jin Y, Heim S, Mandahl N, Johnsson N, Mertens O, Persson B, Salesmark L, Wennerberg J, Mitelman F: Clonal structural chromosome aberration in nonneoplastic cells of the skin and upper aerodigestive tract. Genes Chromosom Cancer 4: 235–240, 1992
74. Pinkel D, Straume T, Gray JW: Cytogenetic analysis using quantitative, high sensitivity fluorescence hybridization. Proc Natl Acad Sci USA 83: 2934–2938, 1986
75. Cremer T, Lichter P, Borde J, Ward DC, Manuelidis L: Detection of chromosome aberrations in metaphase and interphase tumor cells by *in situ* hybridization using chromosome-specific library probes. Hum Genet 80: 235–246, 1988
76. Matsumura K, Kallioniemi A, Kallioniemi O, Chen L, Smith HS, Pinkel D, Gray J, Waldman FM: Deletion of chromosome 17q loci in breast cancer cells detected by fluorescence *in situ* hybridization. Cancer Res 52: 3474–3477, 1992
77. Arnoldus EPJ, Dreef EJ, Noordemeer IA, Verheggen MM, Thierry RF, Peters ACB, Cornelisse CJ, Van der Ploeg M, Raap AK: Feasibility of *in situ* hybridization with

chromosome-specific DNA probes on paraffin wax-embedded tissue. J Clin Pathol 44: 503–513, 1991
78. Hopman AHN, Van Hooren E, Van de Kaa CA, Vooijs PGP, Ramaekers FCS: Detection of numerical chromosome aberrations using *in situ* hybridization in paraffin sections of routinely processed bladder cancers. Mod Pathol 4: 503–513, 1991
79. Dhingra K, Sahin A, Supak J, Kim SY, Hortobagyi G, Hittelman WN: Chromosome *in situ* hybridization on formalin-fixed mammary tissue using non-isotopic, non-fluorescent probes: Technical considerations and biological implications. Breast Cancer Res Treat 23: 201–210, 1992
80. Lee JS, Kim SY, Hong WK, Lippman SM, Ro JY, Gay ML, Hittelman WN: Detection of chromosomal polysomy in oral leukoplakia, a premalignant lesion. J Natl Cancer Inst 85: 1951–1954, 1993
81. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: Comparative genomic hybridization for cytogenetic analysis of solid tumors. Science (Washington, DC) 258: 818–821, 1992
82. Speicher MR, Howe C, Crotty P, Du Manoir S, Costa J, Ward DC: Comparative genomic hybridization detects novel deletions and amplifications in head and neck squamous cell carcinomas. Cancer Res 55: 1010–1013, 1995
83. Knudson AG Jr: Hereditary cancer, oncogenes, and antioncogenes. Cancer Res 45: 1437–1443, 1985
84. Weber JL, May PE: Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44: 388–396, 1989
85. Ah-See KW, Cooke TG, Pickford IR, Soutar D, Balmain A: An allelotype of squamous carcinoma of the head and neck using microsatellite markers. Cancer Res 54: 617–1621, 1994
86. Nawroz H, Van der Riet P, Hruban RH, Koch WM, Ruppert JM, Sidransky D: Allelotype of head and neck squamous cell carcinoma. Cancer Res 54: 1152–1155, 1994
87. El-Naggar AK, Lee MS, Wang G, Luna MA, Goepfert H, Batsakis JG: Polymerase chain reaction-based restriction fragment length polymorphism analysis of the short arm of chromosome 3 in primary head and neck squamous carcinoma. Cancer 72: 881–886, 1993
88. Latif F, Fivosh M, Glenn G, Tory K: Chromosome 3p deletions in head and neck carcinomas. Statistical ascertainment of allelic loss. Cancer Res 52: 1451–1456, 1992
89. Yokoyama S, Yamakawa K, Tsuchiya E, Murata M, Sakiyama S, Nakamura Y: Deletion mapping on the short arm of chromosome 3 in squamous cell carcinoma and adenocarcinoma of the lung. Cancer Res 52: 873–877, 1992
90. Kratzke RA, Simizu E, Kaye FJ: Oncogenes in human lung cancer. Cancer Treat Res 63: 61–85, 1992
91. Hibi K, Takahashi T, Yamakawa K, Ueda R, Sekido Y, Ariyoshi Y: Three distinct regions involved in 3p deletions in human lung cancer. Oncogene 7: 445–449, 1992
92. Killary AM, Wolf ME, Giambernardi TA, Naylor SL: Definition of tumor suppressor locus within human chromosome 3p21–22. Proc Natl Acad Sci USA 89: 10877–10881, 1992
93. Maestro R, Gasparotto D, Vuksavljevic T, Barzan L, Sulfaro S, Boiocchi M: Three discrete regions of deletion in head and neck cancers. Cancer Res 53: 5775–5779, 1993
94. Wu CL, Sloan P, Read AP, Harris R, Thakker N: Deletion mapping on the short arm of chromosome 3 in squamous cell carcinoma of the oral cavity. Cancer Res 54: 6484–6488, 1994
95. Kinzler KW, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB, Smith KJ, Preisinger AC, Hedge P, McKechnie D, Finniear R, Markham A, Groffen J, Boguski MS, Altschul SF, Horii A, Ando H, Miyoshi Y, Miki Y, Nishisho I, Nakamura Y: Identification of FAP locus genes from chromosome 5q21. Science (Washington, DC) 253: 661–665, 1991
96. Boynton RF, Blount PL, Yin J, Brown VL, Huang Y, Tong Y, McDaniel T, Newkirk C, Resan JH, Raskind WH, Haggit RC, Reid BJ, Meltzer SJ: Loss of heterozygosity involving the APC and MCC genetic loci in the majority of human esophageal cancers. Proc Natl Acad Sci USA 89: 3385–3388, 1992
97. Thompson AM, Morris RG, Wallace M, Wyllie AH, Sreel CM, Carter DC: Allele loss from 5q21 (APC/MCC) and 18q21 (DCC) and DCC mRNA expression in breast cancer. Br J Cancer 68: 64–68, 1993
98. Olopade OI, Bohlander SK, Pomykala H, Maltepe E, Van Melle E, Le Beau MM, Diaz MO: Mapping of the shortest region of overlap of deletions of the short arm of chromosome 9 associated with human neoplasia. Genomics 14: 437–443, 1992
99. James CD, Collins VP, Allalunis-Turner MJ, Days RS: Localization of chromosome 9p homozygous deletions in glioma cell lines with markers constituting a continuous linkage map. Cancer Res 53: 3674–3676, 1993
100. Fountain JW, Karayiorgou M, Ernsthoff MS, Kirkwood JM, Vlock DR, Titus-Ernsthoff L, Bouchard B, Vijayasaradhi S, Houghton AN, Lahti J, Kidd VJ, Housman DE, Dracopoli NC: Homozygous deletions within human chromosome band 9p21 in melanoma. Proc Natl Acad Sci USA 89: 10557–10561, 1992
101. Cairns P, Tokino K, Eby E, Sidransky D: Homozygous deletions of 9p21 in primary human bladder tumors detected by comparative multiplex polymerase chain reaction. Cancer Res 54: 1422–1424, 1994
102. Merlo A, Gabrielson E, Askin F, Sidransky D: Frequent loss of chromosome 9 in human primary non-small cell lung cancer. Cancer Res 54: 640–642, 1994
103. Lammie GA, Fantl V, Smith R, Schuuring E, Brookes S, Michalides R, Dickson C, Arnold A, Peters G: D11S287, a putative oncogene on 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. Oncogene 6: 439–444, 1991
104. Berenson JR, Yan J, Micke RA: Frequent amplification of the bcl-1 locus in head and neck squamous cell carcinomas. Oncogene 4: 1111–1116, 1989

105. Sommers KD, Cartwright SL, Schechter GL: Amplifications in the int-2 gene in human head and neck squamous cell carcinomas. Oncogene 5: 915–920, 1990
106. Yoo GH, Xu HJ, Brennan JA, Westra W, Hruban RH, Koch WM, Benedict WF, Sidransky D: Infrequent inactivation of the retinoblastoma gene despite frequent loss of chromsome 13q in head and neck squamous cell carcinoma. Cancer Res 54: 4603–4606, 1994
107. Hall A: *ras* and GAP: Who's controlling whom? Cell 1: 921–923, 1990
108. Bos JL: *ras* oncogenes in human cancer: A review. Cancer Res 49: 4682–4689, 1989
109. Lemoine NR, Mayall ES, Wyllie FS, Williams ED, Goyns M, Stringer B, Wynford-Thomas D: High frequency of *ras* oncogene activation in all stages of human thyroid tumorigenesis. Oncogene 4: 159–164, 1989
110. Vis Vanathan KV, Pocock RD, Summerhayes IC: Preferrential and novel activation of H-*ras* in human bladder carcinomas. Oncogene 3: 77–86, 1988
111. Almoguera C, Shibata D, Forrester K, Martin J, Armheim N, Perucho M: Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. Cell 53: 549–554, 1988
112. Bos JL, Fearon ER, Hamilton JR, Verlaan-de Vries M, Van Boom JH, Van der Eb AJ, Vogelstein B: Prevalence of *ras* gene mutations in human colorectal cancer. Nature (Lond.) 327: 293–297, 1987
113. Rodenhuis S, Slebos RJ, Boot AJM, Evers SG, Mooi NJ, Wagenaar SS, Van Bodegom PC, Bos JL: Incidence and possible clinical significance of K-*ras* oncogene activation in adenocarcinoma of the human lung. Cancer Res 48: 5738–5741, 1988
114. Suzuki Y, Orita M, Shiraishi M, Hayashi K, Sekiya T: Detection of *ras* oncogene mutations in human lung cancers by single strand conformation polymorphism analysis of polymerase chain reaction products. Oncogene 5: 1037–1043, 1990
115. Furth ME, Davis LJ, Fleurdelys B, Scolnick EM: Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular *ras* gene family. J Virol 43: 294–304, 1982
116. Sheng ZM, Barrois M, Klijanienko J, Micheau C, Richard JM, Rio G: Analysis of the c-Ha-*ras*-1 gene for deletion, mutation, amplification and expression in lymph node metastases of human head and neck cancer. Br J Cancer 62: 398–404, 1990
117. Rumsby G, Carter RC, Gusterson BA: Low incidence of *ras* oncogene activation in human squamous cell carcinomas. Br J Cancer 61: 365–368, 1990
118. Saranath D, Chang SE, Bhoite LT, Panchall RG, Kerr IB, Mehta AR, Johnson NW, Deo MG: High frequency mutation in codons 12 and 61 of Ha-*ras* oncogene in tobacco related human oral carcinomas. Br J Cancer 63: 573–578, 1991
119. Kuo MYP, Chang HH, Hahn LJ, Wang JT, Chiang CP: Elevated *ras* p21 expression in oral premalignant lesions and squamous cell carcinomas in Taiwan. J Oral Pathol Med 24: 255–260, 1995
120. Yamamoto T, Nishida T, Miyajima M, Kawai S, Ooi T, Toyoshima K: The erb B gene of the avian erythroblastosis virus is a member of the src gene family. Cell 35: 71–78, 1983
121. Derynck R: Transforming growth factor-α. Cell 54: 593–595, 1988
122. Cohen S, Carpenter G, King L: Epidermal growth factor receptor protein kinase interactions. J Biol Chem 255: 4834–4842, 1980
123. Ushiro H, Cohen S: Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A431 cell membranes. J Biol Chem 255: 8363–8365, 1980
124. Cohen S, Ushiro H, Stoscheck C, Gill GN: Regulation of the epidermal growth factor by phosphorylation. J Cell Biochem 29: 195–208, 1985
125. Velu TJ, Beguinot L, Vass WC, Willingham MC, Merlino GT, Pastan I, Lowy DR: Epidermal growth factor dependent transformation by a human EGF receptor protooncogene. Science (Washington, DC) 238: 1408–1410, 1987
126. Eisbruch A, Blick M, Lee JS, Sacks PJ, Gutterman J: Analysis of epidermal growth factor receptor gene in fresh human head and neck tumors. Cancer Res 47: 3603–3605, 1987
127. Todd R, Donoff BR, Gertz R, Chang A, Chow P, Matossian K, McBride J, Chiang T, Gallagher GT, Wong D: TGF-α and EGF receptor mRNAs in human oral cancers. Carcinogenesis 10: 1553–1556, 1989
128. Maxwell SA, Sacks PJ, Gutterman GU, Gallick GE: Epidermal growth factor receptor protein-tyrosine kinase activity in human cell lines established from squamous carcinomas of the head and neck. Cancer Res 49: 1130–1137, 1989
129. Santini J, Formento JL, Francoval M, Milano G, Schneider M, Dassonville O, Dernard F: Characterization, quantitation and potential clinical value of the epidermal growth factor receptor in head and neck squamous cell carcinomas. Head Neck 13: 132–139, 1991
130. Wong DTW, Biswas DK: Expression of c-erb B oncogene in DMBA-induced hamster oral carcinomas. Oncogene 2: 67–72, 1987
131. Grandis JR, Tweardy DJ: Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 53: 3579–3584, 1993
132. Shin DM, Ro JY, Hong WK, Hittelman WN: Dysregulation of epidermal growth factor receptor expression in premalignant lesions during head and neck tumorigenesis. Cancer Res 54: 3153–3159, 1994
133. Leonard JH, Kearsley JH, Chenevix-Trench G, Hayward NK: Analysis of gene amplification in the head and neck squamous cell carcinomas. Int J Cancer 48: 511–515, 1991
134. Faust JB, Meeker TC: Amplification and expression of the bcl-1 gene in human solid tumor cell lines. Cancer Res 52: 2460–2463, 1992

135. Yoshida T, Miyagawa K, Odagiri H, Sakamoto H, Little PFR, Terada M: Genomic sequence of hst, a transforming gene encoding a protein homologous to fibroblast growth factors and the Int-2 encoded protein. Proc Natl Acad Sci USA 84: 7305–7309, 1987
136. Rosenberg CL, Wong E, Petty EM, Balf AE, Tsujimoto Y, Harris NL, Arnold A: PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. Proc Natl Acad Sci USA 88: 9638–9642, 1991
137. Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM, Arnold AA: A novel cyclin encoded by BCL-1-linked candidate oncogene. Nature (Lond.) 350: 512–515, 1991
138. Kerlseder J, Zeillinger R, Schneeberger C, Czerwenka K, Speiser P, Kubista E, Birnbaum D, Gaudray P, Theillet C: Pattern of DNA amplification at band q13 of chromosome 11 in human breast cancer. Genes Chromosom Cancer 9: 43–48, 1994
139. Williams ME, Gaffey MJ, Weiss LM, Wilczynski SP, Schuuring E, Levine PA: Chromosome 11q13 amplification in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 119: 1238–1243, 1993
140. Jiang W, Kahn SM, Zhou P, Zhang Y, Cacace AM, Infante AS, Doi S, Santella RM, Weinstein IB: Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. Oncogene 8: 3447–3457, 1993
141. Dowdy SF, Hinds PW, Louie K, Reed SI, Arnold A, Weinberg RA: Physical interaction of the retinoblastoma protein with human D cyclins. Cell 73: 499–511, 1993
142. Matsushine H, Ewin ME, Strom DK, Kato J-Y, Hanks SK, Roussel M, Sherr CJ: Identification and properties of an atypical catalytic subunit (p34 p5K-J3/cdk4) for mammalian D type G1 cyclins. Cell 71: 323–334, 1992
143. Bates S, Parry D, Bonetta L, Vousden K, Dickson C, Peters G: Absence of cyclin D/cdk complexes in cells lacking functional retinoblastoma protein. Oncogene 9: 1633–1640, 1994
144. Quelle DE, Ashmun RA, Shurtleff SA, Kato JY, Bar-Sagi D, Roussel MF, Sherr CJ: Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev 7: 1559–1571, 1993
145. Hinds PH, Dowdy SF, Eaton EN, Arnold A, Weinberg RA: Function of a human cyclin gene as an oncogene. Proc Natl Acad Sci USA 91: 709–713, 1994
146. Callender T, El-Naggar AK, Lee MS, Frankenthaler R, Luna MA, Batsakis JG: PRAD-1(CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma. Cancer 74: 152–158, 1994
147. Parise O, Janot F, Guerry R, Fortin A, Luboinski B, Tursz T, Schwaab G, Busson P: Cromosome 11q13 gene amplifications in head and neck squamous cell carcinomas: Relation with lymph node invasion. Int J Oncol 5: 309–313, 1994
148. Michalides R, Van Veelen N, Hart A, Loftus B, Wientjens E, Balm A: Overexpression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck. Cancer Res 55: 975–978, 1995
149. Lebwohl DE, Muise-Helmericks R, Sepp-Lorenzino L, Serve S, Timaul M, Bol R, Borgen P, Rosen NA: A truncated cyclin D1 gene encodes a stable mRNA in a human breast cancer cell line. Oncogene 9: 1925–1929, 1994
150. Roh HJ, Shin DM, Lee JS, Ro JY, Tainsky MA, Hong WK, Hittelman WN: Visualization of int-2 amplification in premalignant lesions during head and neck tumorigenesis (Abstract). Proc Am Assoc Cancer Res 35: 117, 1994
151. Hartwell LH, Kastan MB: Cell cycle control and cancer. Science 266: 1821–1828, 1994
152. Greenblatt MS, Bennett WP, Hollstein M, Harris CC: Mutations in the *p53* tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis. Cancer Res 54: 4855–4878, 1994
153. Brachman DG, Graves D, Vokes E, Beckett M, Haraf D, Montag A, Dumphy E, Mick R, Yandell D, Weichselbaum RR: Occurrence of *p53* gene deletion and human papilloma virus infection in head and neck cancer. Cancer Res 52: 4382–4386, 1992
154. Gusterson BA, Anbazhagan R, Warren W, Midgely C, Lane DP, O'Hare M, Stamps A, Carter R, Jayatilake H: Expression of *p53* in premalignant and malignant squamous epithelium. Oncogene 6: 1785–1789, 1991
155. Field JK, Spandidos SA, Malliri A, Gosney JR, Yiagnisis M, Stell PM: Elevated *p53* expression correlates with a history of heavy smoking in squamous cell carcinomas of the head and neck. Br J Cancer 64: 573–577, 1991
156. Boyle JO, Koch W, Hruban RH, Van der Riet P, Sidransky D: The incidence of *p53* mutations increase with progression of head and neck cancer. Cancer Res 53: 4477–4480, 1993
157. Brennan JA, Mao L, Hruban RH, Boyle JO, Eby YJ, Koch WM, Goodman SN, Sidransky D: Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med 332: 429–435, 1995
158. Shin DM, Kim J, Ro JY, Hittelman J, Roth JA, Hong WK, Hittelman WN: Activation of *p53* gene expression in premalignant lesions during head and neck tumorigenesis. Cancer Res 54: 321–326, 1994
159. Ziegler A, Jonason AS, Leffel DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T, Brash DE: Sunburn and *p53* in the onset of skin cancer. Nature (Lond.) 372: 773–776, 1994
160. Shin DM, Ro JY, Shah T, Hong WK, Hittelman WN: *p53* expression and genetic instability in head and neck multistep tumorigenesis (Abstract). Proc Am Assoc Cancer Res 35: 944, 1994
161. Chung KY, Mukhopadhyay T, Kim J, Casson A, Ro JY, Goepfert H, Hong WK, Roth JA: Discordant *p53* gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. Cancer Res 53: 1676–1683, 1993
162. Nees M, Homann N, Discher H, Andl T, Enders C, Harrold-Mende C, Schulmann A, Bosch FX: Expression of

mutated *p53* occurs in tumor-distant epithelial of head and neck cancer patients: A possible molecular basis for the development of multiple tumors. Cancer Res 53: 4189–4196, 1993

163. Lippman SM, Shin DM, Lee JJ, Batsakis JG, Lotan R, Tainsky MA, Hittelman WN, Hong WK: *p53* and retinoid chemoprevention of oral carcinogenesis. Cancer Res 55: 16–19, 1995
164. Lippman SM, Batsakis JG, Toth BB, Weber RS, Lee JJ, Martin JW, Hays GL, Goepfert H, Hong WK: Comparison of low-dose isotretinoin with beta-carotene to prevent oral carcinogenesis. N Engl J med 328: 15–20, 1993
165. Maxwell SA, Mukhopadhyay T: Transient stabilization of *p53* in non-small cell lung cultures arrested for growth by retinoic acid. Exp Cell Res 214: 67–74, 1994
166. Shin DM, Lee JS, Choi G, Shaw T, Hyne K, Earley C, Shin HJC, Ro JY, Hong WK, Hittelman WN: Prognostic significance of *p53* expression in head and neck squamous cell carcinoma (HNSCC) (Abstract). Proc Am Soc Clin Oncol 13: 293, 1994
167. Clayman GL, El-Naggar AK, Roth LA, Zhang WW, Goepfert H, Taylor DL, Liu TJ: *In vivo* therapy with *p53* adenovirus for microscopic residual head and neck squamous carcinoma. Cancer Res 55: 1–6, 1995
168. Xu L, Davidson BJ, Murty VVVS, Li R-G, Sacks P, Garin-Chesa P, Schantz SP, Chaganti RSK: *p53* gene mutations and CCND1 gene amplification in head and neck squamous cell carcinoma cell lines. Int J Cancer 59: 383–387, 1994
169. Bartkova J, Lukas J, Muller J, Strauss M, Gusterson B, Bartek J: Abnormal patterns of D-type cyclin expression and $G_1$ regulation in human head and neck cancer. Cancer Res 55: 949–956, 1995
170. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer E, Kinzler KW, Vogelstein B: WAF1, a potential mediator of *p53* tumor suppression. Cell 75: 817–825, 1993
171. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ: The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75: 805–816, 1993
172. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D: Induction of WAF1/CIP1 by a *p53*-independent pathway. Cancer Res 54: 3391–3395, 1994
173. Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, Olson EN, Harper JW, Elledge SJ: *p53*-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. Science (Washington, DC) 267: 1024–1027, 1995
174. Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D, Lassar AB: Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science (Washington, DC) 267: 1018–1021, 1995
175. Lee SJ, Ha MJ, Lee J, Trepel JB: *p53*-independent induction of the cyclin-dependent kinase inhibitor p21 in prostate and breast carcinoma cells treated with an inhibitor of the HMG-CoA reductase pathway (Abstract). Proc Am Assoc Cancer Res 36: 581, 1995
176. Hwang MS, Thompson KL, Ahn CH: *p53*-independent induction of $p21^{WAF1}$ and apoptosis by all-*trans*-retinoic acid and 9-*cis* retinoic acid in human prostate cancer cell lines (Abstract). Proc Am Assoc Cancer Res 36: 10, 1995
177. Li R, Waga S, Hannon GJ, Beach D, Stillman B: Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. Nature (Lond.) 371: 534–537, 1994
178. Shiohara M, El-Deiry WS, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen DL, Vogelstein B, Koeffler HP: Absence of WAF1 mutations in a variety of human malignancies. Blood 84: 3781–3784, 1994
179. El-Deiry WS, Tokino T, Waldman T, Oliner JD, Velculescu VE, Burrell M, Hill DE, Healy E, Rees JL, Hamilton SR, Kinzler KW, Vogelstein B: Topological control of $p21^{waf1/cip1}$ expression in normal and neoplastic tissues. Cancer Res 55: 2910–2919, 1995
180. Lipkin M, Bell B, Sherlock P: Cell proliferation kinetics in the gastrointestinal tract of man: I. Cell renewal in colon and rectum. J Clin Invest 42: 767, 1963
181. Lipkin M, Sherlock P, Bell B: Cell proliferation kinetics in the gastrointestinal tract of man: II. Cell renewal in stomach, ileum, colon and rectum. Gastroenterology 45: 721, 1963
182. Serrano M, Hannon G, Beach D: A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. nature (Lond.) 366: 704–707, 1993
183. Van der Riet P, Nawroz H, Hruban RH, Corio R, Tokino K, Koch W, Sidransky D: Frequent loss of chromosome 9p21–22 early in head and neck cancer progression. Cancer Res 54: 1156–1158, 1994
184. Kamb A, Gruis NA, Weaverfeldhaus J, Liu QY, Harshman K: A cell cycle regulator potentially involved in genesis of many tumor types. Science 264: 436–440, 1994
185. Cairns P, Mao L, Merlo A, Lee DJ, Schwab D, Eby Y, Tokino K, Van der Riet P, Blaugrund JE, Sidransky D: Low rate of p16 (MTS1) mutations in primary tumors with 9p loss. Science (Washington, DC) 265: 415–417, 1994
186. Zhang SY, Klein-Szanto AJP, Sauter ER, Shafarenko M, Mitsunaga S, Nobori T, Carson DA, Ridge JA, Goodrow TL: Higher frequency of alterations in the p16/CDKN2 gene in squamous cell carcinoma cell lines than in primary tumors of the head and neck. Cancer Res 54: 5050–5053, 1994
187. Lydiatt WM, Murty VVVS, Davidson BJ, Xu L, Dyomina K, Sacks PG, Schantz SP, Chaganti RSK: Homozygous deletions and loss of expression of the CDKN2 gene occur frequently in head and neck squamous cell carcinoma cell lines but infrequently in primary tumors. Genes Chromosom Cancer 13: 94–98, 1995
188. Spruck CH, Gonzales-Zulueta M, Shibata A, Simoneau AR, Lin MF, Gonzales F, Tsai YC, Jones PA: p16 gene in uncultured tumors. Science (Washington, DC) 265: 415–416, 1994

189. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger P, Baylin SB, Sidransky D: 5′ CpG island methylation is associated with transciptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. Nature Med 1: 686–692, 1995
190. Bravo R, Frank R, Blundell PA, MacDonald-Bravo H: Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. Nature (Lond.) 326: 515–517, 1987
191. Baserga R: Growth regulation of the PCNA gene. J Cell Sci 98: 433–436, 1991
192. Celis JE, Celis A: Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: Subdivision of S phase. Proc Natl Acad Sci USA 82: 3262–3266, 1985
193. Shin DM, Voravud N, Ro JY, Lee JS, Hong WK, Hittelman WN: Sequential upregulation of proliferating cell nuclear antigen in head and neck tumorigenesis: A potential biomarker. J Natl Cancer Inst 85: 971–978, 1993
194. Huang WY, Coltrera M, Schubert M, Morton T, Truelove E: Histopathologic evaluation of proliferating cell nuclear antigen (PC10) in oral epithelial hyperplasias and premalignant lesions. Oral Surg Oral Med Oral Pathol 78: 748–754, 1994
195. Gorgoulis V, Zoumpourlis V, Rassidakis G, Karameris A, Barbatis C, Spandidos DA, Kittas C: Molecular analysis of *p53* gene in laryngeal premalignant and malignant lesions: *p53* protein immunohistochemical expression is positively related to proliferating cell nuclear antigen labelling index. Virchows Arch 426: 339–344, 1995
196. Girod SC, Pape HD, Krueger GR: *p53* and PCNA expression in carcinogenesis of the oropharyngeal mucosa. Eur J Cancer B Oral Oncol 30b: 419–423, 1994
197. Jetten AM: Multistep process of squamous differentiation of tracheobronchial epithelial cells. Role of retinoids. Dermatologica 175: 37–44, 1987
198. Moll R, Franke WW, Schiller DL: The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 31: 11–24, 1982
199. Kim KH, Schwartz F, Fuchs E: Difference sin keratin synthesis between normal epithelial cells and squamous cell carcinomas are mediated by vitamin A. Proc Natl Acad Sci USA 81: 4280–4284, 1984
200. Rutten AAJJL, Bruyntjes JP, Ramaekers FCS: Effect of cigarette smoke condensate and vitamin A depletion on keratin expression patterns in cultured hamster tracheal epithelium: An immunohistomorphological study using monoclonal antibodies to keratins. Virchows Arch [B] 56: 111–117, 1989
201. Lindberg K, Rheinwald JG: Suprabasal 40 kd keratin (K19): Expression as immunohistological marker of premalignancy in oral epithelium. Am J Pathol 134: 419–426, 1989
202. Gimenez-Conti IB, Shin D, Bianchi AB, Roop DR, Hong WK, Conti CJ, Slaga TJ: Changes in keratin expression during 7,12-dimethylbenz(a) anthracene-induced hamster cheek pouch carcinogenesis. Cancer Res 50: 4441–4445, 1990
203. Copper MP, Braakhuis BJM, De Vries N, Van Dongen GAMS, Nauta JJ, Snow G: A panel of biomarkers of carcinogenesis of the upper aerodigestive tract as potential intermediate endpoints in chemoprevention trials. Cancer 71: 825–830, 1993
204. Thatcher SM: Purification of keratinocyte transglutaminase and its expression during squamous differentiation. J Invest Dermatol 92: 578–584, 1989
205. Said JW, Nash G, Sassoon AF, Shintaku IP, Banks-Schlegel S: Involucrin in lung tumors: A specific marker for squamous differentiation. Lab Invest 49: 563–568, 1983
206. Itoiz ME, Conti CJ, Gimenez-Conti IB, Lafranchi HE, Fernandez-Alonso GI, Klein-Szanto AJP: Immunodetection of involucrin in lesions of the oral mucosa. J Oral Pathol 15: 205–208, 1986
207. Lippman SM, Lee JS, Lotan R, Hong WK: Chemoprevention of upper aerodigestive tract cancers: A report of the Upper Aerodigestive Cancer Task Force workshop. Head Neck Surg 12: 5–20, 1990
208. Lotan R: Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochim Biophys Acta 605: 33–91, 1980
209. Lippman S, Toth B, Batsakis J: The evaluation of biologic markers as intermediate endpoints in a chemoprevention trial of 13-cis-retinoic acid (13cRA) in human premalignant oral lesions (Abstract). Proc Am Soc Clin Oncol 8: 168, 1989
210. Sellers TA, Potter JD, Bailey-Wilson JE, Rich SS, Rothschild H, Elsta RC: Lung cancer detection and prevention: Evidence for an interaction between smoking and genetic predisposition. Cancer Res 52: 2694–2697, 1992
211. Harris CC: Interindividual variation among humans in carcinogen metabolism, DNA adduct formation and DNA repair. Carcinogenesis 10: 1563–1566, 1989
212. Hagmar L, Brogger A, Hansteen IL, Heim S, Hogstedt B, Knudsen L, Lambert B, Linnainmaa K, Mitelman F, Nordenson I, Reuterwall C, Salomaa S, Skerfving S, Sorsa M: Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage. Cancer Res 54: 2919–2922, 1994
213. Hsu TC: Genetic predisposition to cancer with special reference to mutagen sensitivity. In Vitro Cell Dev Biol 23: 591–603, 1987
214. Spitz MR, Fueger JJ, Beddingfield NA, Annegers JF, Hsu TC, Newell GR, Schantz SP: Chromosome sensitivity to bleomycin-induced mutagenesis, an independent risk factor for upper aerodigestive tract cancer. Cancer Res 49: 4626–4628, 1989
215. Spitz MR, Fueger JJ, Halabi S, Schantz SP, Sample D, Hsu TC: Mutagen sensitivity in upper aerodigestive tract cancer: A case control analysis. Cancer Epidemiol Biomarkers Prev 2: 329–333, 1993
216. Spitz MR, Hoque A, Trizna Z, Schantz SP, Amos CI, King

TM, Bondy ML, Hong WK, Hsu TC: Mutagen sensitivity as a risk factor for second malignant tumors following malignancies of the upper aerodigestive tract. J Natl Cancer Inst 86: 1681–1684, 1994

217. Cloos J, Braakhuis BJ, Steen I, Copper MP, De Vries N, Nauta JJ, Snow GB: Increased mutagen sensitivity in head-and-neck squamous-cell carcinoma patients, particularly those with multiple primary tumors. Int J Cancer 56: 816–819, 1994
218. Dave BJ, Hsu TC, Hong WK, Pathak S: Nonrandom distribution of mutagen-induced chromosome breaks in lymphocytes of patients with different malignancies. Int J Oncol 5: 733–740, 1994
219. McGlynn KA, Buetow KH: Metabolic and H-*ras* polymorphisms in genetic susceptibility. Cancer Bull 46: 220–227, 1994
220. Lippman SM, Kessler JF, Meyskens FL: Retinoids as preventive and therapeutic anticancer agents. Cancer Treat Rep 71: 391–405, 1987
221. Moon TE, Cartmel B, Levine N, Arizona Skin Cancer Study Group: Chemoprevention and etiology of non-melanoma skin cancers, program, and abstracts. 17th Annual Meeting of the American Society of Preventive Oncology, Tucson, March 20–23, 1993
222. Meyskens FL, Surwit E, Moon TE, Cilders JM, Davis JR, Dorr RT, Johnson CS, Alberts DS: Enhancement of regression of cervical intraepithelial neoplasia II (moderate dysplasia) with topically applied all-*trans*-retinoic acid: A randomized trial. J Natl Cancer Inst 86: 539–543, 1994
223. Pastorino U, Ifante M, Maioli M: Adjuvant treatment of stage I lung cancer with high-dose vitamin. Am J Clin Oncol 11: 1216–1222, 1993
224. Kraemer KH, DiGiovanna JJ, Moshell AN Tarone RE, Deck GL: Prevention of skin cancer in xeroderma pigmentosum with the use of oral isotretinoin. N Engl J Med 318: 1633–1637, 1988
225. Smith MA, Parkinson DR, Cheson BD, Friedman MA: Retinoids in cancer therapy. J Clin Oncol 10: 839–864, 1992
226. Gudas LJ, Sporn MB, Roberts AB: Cellular biology and biochemistry of the retinoids. In: Sporn MB, Roberts AB, Goodman DS (eds) The Retinoids: Biology, Chemistry, and Medicine. Reven Press, New York, 1994, pp 443–520
227. Lotan R: Retinoids and squamous cell differentiation. In: Hong WK, Lotan R (eds) Retinoids in Oncology. Marcel Dekker, New York, 1993, pp 43–72
228. Borden EC, Lotan R, Levens D, Young CW, Waxman S: Differentiation therapy of cancer: laboratory and clinical investigations (meeting report). Cancer Res 53: 4109–4115, 1993
229. Leroy P, Krust A, Kastner P, Mendelsohn C, Zelent A, Chambon P: Retinoic acid receptors. In: Morriss-Kay G (ed) Retinoids in Normal Development and Teratogenesis. Oxford University Press, New York, 1992, pp 7025
230. Huang M, Ye Y, Chen S, Chai J, Lu J, Zhoa L, Gu L, Wang Z: Use of all-*trans*-retinoic acid in the treatment of acute promyelocytic leukemia. Blood 72: 567–572, 1988
231. Moasser MM, DeBlasio A, Dmitrovsky E: Response and resistance to retinoic acid are mediated through the retinoic acid nuclear receptor gamma in human teratocarcinomas. Oncogene 9: 833–840, 1994
232. Zou CP, Clifford JL, Xu XC, Sacks PG, Chambon P, Hong WK, Lotan R: Modulation by retinoic acid (RA) of squamous cell differentiation, cellular RA-binding proteins, and nuclear RA receptors in human head and neck squamous cell carcinoma cell lines. Cancer Res 54: 5479–5487, 1994
233. Xu XC, Ro JY, Lee JS, Shin DM, Hong WK, Lotan R: Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. Cancer Res 54: 3580–3587, 1994
234. Lotan R, Sozzi G, Ro J, Lee J, Pastorino U, Pilotti S, Kurie J, Hong WK, Xu X: Selective suppression of retinoic acid receptor β (RAR β) expression in squamous metaplasia, and in non-small cell lung cancers (NSCLC) compared to normal bronchial epithelium (Abstract). Proc Am Soc Clin Oncol 14: 61, 1995
235. Hu L, Crowe DL, Rheiwald JG, Chambon P, Gudas LJ: Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. Cancer Res 51: 3972–3981, 1991
236. Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG: High frequency of retinoic acid receptor beta abnormalities in human lung cancer. Oncogene 6: 1859–1868, 1991
237. Lotan R, Xu X-C, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK: Suppression of retinoic acid receptor β in premalignant oral lesions and its upregulation by isotretinoin. N Engl J Med 332: 1405–1410, 1995

*Address for offprints:*
D.M. Shin, Box 80, Department of Thoracic/Head and Neck Medical Oncology, M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas, 77030, USA