Yonsei Medical Journal
Vol. 35, No. 2, 1994

□ Review Article □

Biomarkers in Chemoprevention for Upper Aerodigestive Tract Tumors

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A chemopreventive approach to cancers of the upper aerodigestive tract (including those of head and neck and lung) to reduce the incidence and mortality rates for these cancers has become an important strategy because therapies such as surgery, radiation, and chemotherapy have only marginally improved the five-year survival rate over the last two decades. However, chemopreventive trials have been hampered by serious feasibility problems, including high cost, the requirement of large numbers of patients, and long-term follow-up necessary to determine cancer incidence, which served as the study end point. Thus, the use of biomarkers, the identification of which would serve as an intermediate end point of the study has recently emerged as a subject of great interest. To try to understand the process of tumorigenesis from normal tissues through the premalignant tissue stage to malignant lesions, there has recently been a search for genetic and/or phenotypic changes that qualify as candidates for biomarkers. These candidates include genomic markers, certain specific genetic markers (such as oncogenes, growth factors and their receptors, and tumor suppressor genes), cell proliferation markers, and cell differentiation markers. This review covers genomic markers (including micronuclei and specific chromosomal alterations) and specific genetic markers (such as the ras gene family, the myc family, erb B1, int-2/hst-1, and the p53 tumor suppressor gene). As a consequence of genetic alteration, we also reviewed cell proliferation markers such as proliferating cell unclei antigen (PCNA) and the squamous cell differentiations markers, including keratins, involucrin, and transglutaminase 1. These biomarker candidates are important adjuncts to the development of the new chemopreventive agents and to the rational design of future intervention trials. However, it should be emphasized that these biomarkers must first be validated in clinical trials; only then can they replace cancer incidence as the sole end point in chemoprevention trials.

Key Words: Biomarkers, chemoprevention, head and neck and lung cancers, tumorigenesis

Epithelial cancers of the upper aerodigestive tract, including head and neck and lung cancers, are an increasingly important public health problem throughout the world.

Despite the advances in surgery, radiotherapy, and chemotherapy over the last two decades, the five-year survival rates associated with these cancers have improved only marginally. New research directions are clearly indicated. There has been renewed interest in chemoprevention as a means of reducing the incidence of and mortality from these cancers (Meyskens 1990; Boone *et al.* 1990; Hong *et al.* 1990). Unfortunately, chemopreventive approaches have faced serious feasibility problems associated with the

Received May 10, 1994

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Supported in part by National Health Institute grant CA-52501 and American Cancer Society grant ACS91-271

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conduct of a large number of randomized phase III chemoprevention trials. Investigators have been forced to rely on cancer incidence as the study end point for determining a regimen's preventive efficacy. As a result, these trials have required numbers of subjects and long-term follow-up, thus making them very costly. All this has prompted a recent great surge of interest in defining "biomarkers" associated with the specific stages of carcinogenesis with the goal of using detection of these markers as intermediate end points in chemoprevention trials, thus making the trials shorter and less cost (Lippman *et al.* 1990; Schatzkin *et al.* 1990). This concept has been well elucidated by Zelen (1988) who stated that the use of biomarkers detection to define the intermediate end points would make prevention trials feasible.

Interest in systemic therapies to prevent cancer in the aerodigestive tract has come from an understanding that the entire epithelial lining of the tract has in common a repeated exposure to carcinogenic substances and the resulting increase in cancer risk. This concept of the entire epithelium as a "condemned mucosa" was first described by Slaughter *et al.* (1953), who coined the term "field cancerization" to described the diffuse histologic abnormalities and multifocal nature of squamous cell carcinomas of the head and neck. In cancers of the aerodigestive tract this hypothesis is supported clinically by the frequent association of these tumors with premalignant lesions (i.e., oral leukoplakia (Silverman *et al.* 1984) or bronchial metaplasia and/or dysplasia (Auerback *et al.* 1961) in the field or area at risk and by the synchronous or metachronous development of multiple primary tumors (Lippman *et al.* 1989). Another important concept in aerodigestive tract cancer is the multistep carcinogenic process (Farber *et al.* 1984). The driving force behind this process is thought to be genetic damage caused by continuous exposure of tissues to carcinogens, as evinced by increased numbers of micronuclei in high-risk tissue and premalignant lesions which often are present in this setting (Stich, 1987). However, these genetic alterations can also be driven by certain specific gene alterations, such as oncogenes (i.e., *ras, myc*) and growth-factors and growth-factor receptor genes (i.e., epidermal growth factor (EGF), EGF receptor (EGFR), transforming-growth factor-α, and tumor suppressor genes. These genetic markers or specific genes may eventually result in changes of phenotypic markers, including dysregulation of cell proliferation and differentiation. These biomarker candidates are summarized in table 1. The genetic alterations and phenotypic consequences can be visualized as histologic transitions from normal epithelium to hyperplasia to metaplasia/dysplasia and then to frank malignant tumors.

Table 1. Candidates of biomarkers for upper aerodigestive tract tumors

Genomic Markers (General)
Nuclear aberrations (e.g., micronuclei)
DNA content and flow cytometry
Chromosomal alterations
Specific Genetic Markers
Oncogene alterations [ras family, *myc* family, *erb* family(erbB1, erbB2/Her-2/neu)]
Src family(*Src, lck*), retinoic acid receptors
Tumor suppressor genes [*p53* gene, retinoblastoma *(rb)* gene, 3p genes(unidentified)
Retinoic Acid receptors(RAR-α, RAR-β, RAR-γ, RXRs)
Cell proliferation Markers
Mitotic frequency(MPM-2)
Thymidine labeling index
Nuclear antigens (e.g., PCNA, DNA-polymee-α, Ki-67)
Polyamines, ornithine decarboxylase
Cell differentiation Markers
Cytokeratins
Transglutaminase type I
Involucrin

BIOMARKER SELECTION

The process of developing biomarkers, from the laboratory to their clinical application in humans, requires new insight and disciplines (Stockman *et al.* 1992). The new paradigm of carcinogenesis is a multistage model that has potential genetic and/or phenotypic markers at each stage. Tumor initiation is characterized by

stepwise genetic changes in cells from normal to malignant cells. To elucidate the multistep nature of the carcinogenesis process many investigators have worked out elegant models (Pitot 1992; Harris *et al.* 1988; Slaga 1989) in which cellular or genetic alterations may precede invasive malignancy and can thus serve as an intermediate indicators of the carcinogenic process. Biomarkers, then, can be defined as measurable markers of cellular or molecular genetic events that are associated with specific stages of carcinogensis and that can serve as intermediate end points in clinical trials. Because the efforts to search for biomarkers in chemoprevention is just beginning, no individual biomarker or pattern of biomarkers has yet been validated through prevention trials as a conclusive predictor of carcinogenic transformation, though several validation studies are ongoing or in planning. Toward this end, Lippman *et al* (1990) proposed the following criteria to establish biomarkers for tobacco-related epithelial carcinogenesis: (a) differential expression of the biomarker in normal and high-risk tissues, (b) ability to analyze the biomarker in small tissue specimens, (c) a quantitative degree or pattern correlating the biomarker with the stage of carcinogenesis, and (d) the availability or preclinical of eary clinical data supporting the modulation of the biomarker by study agents.

Oncogene activation and/or inactivation of tumor suppressor genes may emerge as markers of early-stage carcinogenesis, although these events may also occur at later stages of tumor progression (Harris it *et al.* 1990). The activation of certain oncogenes (i.e., *ras, myc, neu/her-2, ras, fur*) has been associated with the development of lung cancer (Harris *et al.* 1990). However, the activation sequence of these genes, which would define which oncogenes are involved with the earliest stage of carcinogenesis, has not been well established. K-*ras* expression has been detected frequently in bronchial adenocarcinoma tumor tissue (Pulciani *et al.* 1982; Rodenhuis *et al.* 1987), and has been associated with shortened patient survival in both early and advanced stages of decease (Slebos *et al.* 1990; Mitsudomi *et al.* 1991). Over expression of the c-*myc* gene has been associated with growth deregulation and with loss of terminal differentiation in squamous cell (Field *et al.* 1989; Sarnath *et al.* 1989) and small-cell tumors (Johnson *et al.* 1987; Birrer *et al*, 1989; Yokota *et al.* 1988). Using probes to detect an allelic deletion and restriction fragment length polymorphisms for specific chromosomal regions, loss of heterozygosity has been found frequently on chromosomes 3p (100%), 13q (91%), and 17p (100%) in patients with small cell lung cancers (SCLCs) (Yokoda *et al.* 1987). The karyotypes in non-small cell lung cancers (NSCLC) are very complex, but the recurrent loss of chromosomes 17p, 3p, and 11p (in 67%, 57%, and 47% of cases, respectively) suggests that these regions are "hot spots" for genetic alterations (Miura *et al.* 1990). Other candidate regions with breakpoints indicating potential recessive oncogenes include 1q, 3q, 5q, 7p, 16q24, and 21p (Whang-Peng *et al.* 1991).

Since carcinogenesis begins with certain specific gene alterations and/or general genomic alterations that later result in phenotypic changes such as dysregulation of cell proliferation and differentiation, we place biomarkers having potential chemopreventive utility in four general classes: general genomic markers, specific genetic markers, proliferation markers, and differentiation markers. These biomarkers can reflect relatively early site-specific carcinogenic changes, such as those in the *erbB1* gene in oral carcinogenesis and the K-*ras* gene in bronchial carcinogenesis (Lippman *et al.* 1990). Additionally, these markers can represent changes that are not site specific or that occur over extended periods of time.

GENOMIC MARKERS (GENERAL)

Micronuclei

The most widely studied genomic marker in earlier chemoprevention trials was the increased numers of micronuclei (Lippman *et al.* 1990; Stich *et al.* 1988; Stich *et al.* 1988, Rosin *et al.* 1987, Lippman *et al.* in press,). Because micronuclei are easy to find and quantify, they are the most studied of all potential intermediated end-points biomarkers in chemoprevention trials. Micronuclei are chromosome and chromatid fragments formed in proliferating cells during clastogenic

events such as DNA damage by carcinogens. The micronuclei in the aerodigestive tract epithelium are formed in the basal layer, which gives rise to suprabasal cells that migrate to the epithelial surface and can eventually be detected in exfoliated cells. The presence and frequency of micronuclei in tissues are believed to quantitatively reflect ongoing DNA damage. A series of trials has shown that high numbers of micronuclei correlate with a cancer risk to target tissues in individuals (e.g., smokers) at high risk for the development of head and neck and lung cancer (Stich *et al.* 1988).

Because increased micronuclei numbers fits all of the biomarker selection criteria, it seems obvious that micronuclei would be a useful biomarker to serve as an intermediate end point in upper areodigestive tract cancer chemopreverntion trials. A critical analysis of the extensive data on this marker, however, reveals problems with its use as the only marker (Lippman *et al.* in press). Stich *et al.* (1988) studied chemoprevention in groups of such high-carcinogenic exposure to betel nuts, a uniform life style and dietary habits, and showed consistently the elevated numbers of micronuclei in their oral cavities. These investigators reported that retinol and beta-carotene each suppressed micronuclei formation in more than 90% of lesions; however, both the rates of clinical tumor response and the rates of suppression of new lesions differed greatly between the two agents. Thus, a single biomarker without other supporting data may not be useful in screening for active chemopreventive drugs. Despite these problems, studies using micronuclei as a biomarker have contributed to the early development of natural compounds (i.e., retinoids and carotenoids) as potential chemopreventive agents, and micronuclei frequency may still have a role as a biomarker included in a series or panel with others specific genetic or phenotypic markers.

Chromosomal alterations in carcinogenesis

Head and neck cancer is a unique model for the study of tumorigenesis and the development of biomarkers for several reasons. First, head and neck cancer has been proposed to be representative of the process of field cancerization because the entire aerodigestive tract epithelium is repeatedly exposed to carcinogenic insults (e.g., from tobacco and alcohol), thus placing the entire epithelial field at risk for tumor development (Gluckman *et al.* 1980). Clinical evidence for this hypothesis is the high frequency of multiple primary neoplasms in the aerodigestive tract and the increased risk of second primary tumors (Auerbach *et al.* 1961; Lippman *et al.* 1989). Second, head and neck cancer is thought to be represent a multistep tumorigenesis process whereby a series of events occur prior to tumor development (Shibuya *et al.* 1987). This process is evidenced by the presence of premalignant lesions adjacent to tumor (Mitelman, 1988). Although there is clinical and histologic evidence of these processes, biomarkers for these processes are lacking. Therefore, our laboratory investigated the genetic changes in the tissue at risk by using chromosome-specific probes and *in situ* hybridization. Although a variety of cytogenetic changes have been described in head and neck and lung cancers (Jin *et al.* 1990; Osella *et al.* 1992); a comprehensive study of specific chromosomal changes has been limited by impediments common to cytogenetic studies of solid tumors, (i.e., the low frequency of mitotic figures from direct preparations, suboptimal chromosomal alterations, and multiple complexity of cytogenetic changes) (Tessier, 1989). Identification of chromosomal changes in premalignant lesions is technically more difficult with conventional cytogenetic procedures and has thus seldom been repoted (Lee *et al.* 1987; Sozzi *et al.* 1991; Mertens *et al.* 1992). The recent development of *in situ* hybridization (ISH) techniques has allowed detection of chromosomal abnormalities directly in interfaces cells (Pinkel *et al.* 1986; Cremer *et al.* 1988; Hopman *et al.* 1986; Van Dekken *et al.* 1990; Reid *et al.* 1992). This method has now been applied to tumor cell lines or dissected tumor materials from many different types of tumors (Cremer *et al.* 1988; Hopman *et al.* 1988; Hopman *et al.* 1991; Matsumura *et al.* 1992). More recently, ISH has been adapted for use on formalin-fixed, paraffin embedded tissues sections by using non-isotopic, chromosome-specific DNA probes (Emmerich *et al.* 1989; Arnolds *et al.* 1991; Hopman *et al.* 1991; Dhingra *et al.* 1992; Kim *et al.* 1993). This tech-

niques allows direct visualization of chromosomal changes in normal tissue, premalignant lesions, and tumor tissue with preservation of tissue architecture.

To visualize the accumulation of genetic alterations during head and neck tumorigenesis and to determine the extent of the genetically altered field, we probed 25 squamous cell carcinomas of the head and neck and there adjacent premalignant lesions to detect numerical chromosome aberrations by nonisotopic ISH using chromosome-specific centromeric DNA probes for chromosomes 7 and 17. Normal control oral epithelium from cancer-free nonsmokers showed no chromosome polysomy (i.e., cells with three or more chromosome copies), whereas histologically normal epithelium adjacent to tumors showed squamous cell polysomies of chromosomes 7 and 17 (Voravud *et al.* 1993). Moreover, the number of cells with polysomy increased as the tissues progressed from histologically normal epithelium to hyperplasia to dysplasia and then to cancer. The presence of chromosomal abnormalities in histologically normal and precancerous tissues adjacent to the tumor samples supports the concept of field cancerization (Voravud *et al.* 1993). Likewise, the finding of progressive genetic changes as the tumor develops supports the concept of multistep tumorigenesis in the head and neck region. These chromosome number changes in premalignant lesions near the head and neck tumor is not unexpected, as most of the carcinogens that cause upper aerodigestive tract tumors are known to cause chromosomal abnormalities (Lofroth, 1989; Kao-Shan *et al.* 1987). In fact, many other studies have reported increased number of micronuclei at various sites in the aerodigestive tract both at sites exposed to the varous related carcinogens and at sites harboring premalignant lesions (Stich *et al.* 1988). Individuals with head and neck cancer also have demonstrated increased in vitro sensitivity to chromosome-damaging agents, especially in those individuals in whom second primary tumors develop (Schantz *et al.* 1990; Hsu *et al.* 1991).

One of the goals of this study was to identify genetic biomarkers that might be useful for assessing the probability of tumor development in hign-risk patients and for serving as an intermediate end point in chemoprevention studies. The findings of chromosomal polysomies in histologically normal epithelium adjacent to tumors and the increased chromosome polysomies as the tissue progressed through various stages to carcinoma suggest that the degree of chromosomes polysomies might be such a good genetic biomarker. The advantage of such a biomarker is that it permits the sensitive detection of infrequent events that reflect accumulated genetic damage or genomic instablity which is difficult to detect by bulk analysis (e.g., DNA content analysis).

SPECIFIED GENETIC MARKERS

Ras gene family

A significant proportion of human tumors from various sites in the body have been shown to contain activated oncogenes of the ras family (Harvey-ras, Kirsten-*ras*, N-*ras*) (Fugita *et al.* 1994; Eva *et al.* 1983; Santos, 1984). Oncogenes in this family are forms of the germ-line protooncogenes, with specific point nutations that when transfected onto NIH/3T3 murine fibroblasts, introduce foci of morphologically cells (Shih *et al.* 1979; Der *et al.* 1982; Goldfarb *et al.* 1982). Normal *ras* genes code for proteins of molecular weights of approximately 21,000 that have guanine nucleotide-binding activity and are to hydrolyze guanine triphospate (GTP) (Papageorge *et al.* 1982). The *ras* proteins possess intrinsic GTPase activity that eventually leads to their inactivation, but this inactivations is greatly enhanced by a second protein, called GAP(GTPase activity protein) (McCormick 1989). GAP has been known to bind to the domain that is involved in the transduction of the *ras* gene signal, the "effector domain" of p21 *ras* (McCormick 1990; Hall 1990).

Mutationally activated *ras* genes have been found in a wide range of human tumors (Bos 1989). With some reasons, K-*ras* is particularly associated with adenocarcinomas and has been reported to be activated in pancreatic cancers (Almoguera *et al.* 1988; Smit *et al.* 1988), colorectal cancers (Bos *et al.* 1987; Forrester *et al.* 1987) and adenocarcinoma of the lung (Slebos *et al.*

1990; Rodenhuis *et al.* 1987; Rodenjuis *et al.* 1988). In contrast, SCLC is not associated with an activated *ras* oncogene (Mitsudomi *et al.* 1991). K-*ras* gene activation has also reported to predict an unfavorable outcome; identifying some patients who have a poor prognosis despite apparently successful surgery for stage I or II tumors (Slebos *et al.* 1990). *Ras* gene exporession was studied in squamous cell carcinomas of head and neck and lung tissues by immunohistochemistry and was shown to correlate with a degree of tumor differentiation with clinical disease stages, and with clinical outcomes (Azuma *et al.* 1987). In that study, 59 of 121 tumor samples reacted to the monoclonal antibody Y13-259 raised against the protein encoded by the Harvey murine sarcoma virus (Furth *et al.* 1982), while samples from oral leukoplakia and normal mucosa did not react, indicating that they did express this protein. Azuma *et al.* (1987) also reported that the expression of *ras* was associated with a poor prognosis. A few studies have reported on *ras* gene mutations in premalignant lesions. In colonic adenomas from patients with familial polyposis coli the K-*ras* mutations were found in nontumorous adenomatous polyps, indicating that K-*ras* mutations could be a useful marker in colonic tumorigenesis (Farr *et al.* 1988). In patients with colorectal tumors, the K-*ras* gene mutations were detectable in DNA purified from the stool samples (Sidransky *et al.* 1992). The level of H-*ras* gene expression was examined at different stages of 7, 12-dimethybenz(a)anthracene (DMBA)-induced tumor development in hamster buccal pouch epithelium (Husain *et al.* 1989). The results demonstrated that the H-*ras* gene was overexpressed at a very early stage of tumor development and that this overexpression persisted throughout the tumorigenesis process in a stage-specific manner (Husain *et al.* 1989). In conclusion, *ras* gene activation can be a potential candidate for a biomarker in epithelial tissues during tumorigenesis, although further studies need to be performed in human samples.

ErbB1 or epidermal growth factor receptor (EGFR) gene

The *erbB1* oncogene was initially discovered as one of the two oncogenes carried by the avian erythroblastosis virus (Vennestrom *et al.* 1982). The corresponding proto-oncogenes were found to encode a membrane-associated tyrosine kinase protein that was eventually identified as the receptor for EGF (Lin *et al.* 1984; Ullrich *et al* . 1984; Xu *et al.* 1984). As these receptors bind their respective ligands, the tyrosine kinase activity of each is stimulated several-fold, as indicated by enhanced autophosphorylation of the receptor, increased the phosphorylation of exogenous substrates *in vitro*, and elevated phosphorylation of the tyrosine residues of several proteins *in vitro* (Sachs *et al.* 1988). To examine the possibility that EGFR levels were higher in the tumor than in the corresponding controls. They also found a significant direct correlation between EGFR levels and tumor size and stage. Another study showed expression of the Ki-67 antigen, EGFR, transferrin receptor (TFR), and DNA ploidy in 42 fresh tumor samples from head and neck carcinomas. This study suggested that EGFR and TFR are widely distributed, especially on proliferating cells at the invading tumor margins (Kearsley *et al.* 1990). In addition, there was a close spatial correlation between cells that expressed EGFR and TFR and those that expressed KI-67 antigen. Further follow-up is necessary to determine whether these parameters will be important prognostic factors (Kearsley *et al.* 1990). The *EGFR* gene has also been found to be amplified in NSCLC (up to 20% in squamous cell types) (Cline *et al.* 1984; Berger *et al.* 1987; Shiraishi *et al.* 1989), whereas EGFR protein overexpression has been shown in many NSCLC cells approximately 90% of squamous cell types. (Hendler *et al.* 1986; Cerny *et al.* 1986). However, *EGFR* amplification or protein overexpression has not been seen in SCLC cells. The overexpression of the EGFR protein may reflect the development of an autocrine loop, as most epithelial cells including NSCLC cells require *EGF* for their growth (Johnson *et al.* 1990). These findings have led us to develop clinical trials that use monoclonal antibody against the EGFR in the treatment of NSCLC (Mendelsohn *et al.* 1988; Perez-Soler *et al.* 1994).

The expression of EGFR in premalignant lesions, however, has not been well studied. To determine whether EGFR is over-expressed in premalignant lesions, we recently examined 36

head and neck squamous cell cancers along with their adjacent premalignant lesions and normal epithelia. Using a monoclonal anti-EGFR antibody for immunohistochemical analysis on paraffin-embedded tissue sections, we examined EGFR expression and quantitated it by computer-assisted image analysis (Shin *et al.* 1993). The EGFR expression was significantly higher in the normal tissue adjacent to tumors than it was in control samples ($p=0.021$), which were never exposed to tobacco and/or alcohol. The finding of higher EGFR expression even in normal epithelium adjacent to tumors supports the hypothesis of field cancerization addressed earlier (Slaughter *et al.* 1953). We also found that there is another increment of EGFR expression at the transition from dysplastic lesions to squamous cell carcinomas, in two thirds of the samples examined ($p=0.001$). These results thus indicate that EGFR expression could be an important regulatory marker in the multistep process of head and neck carcinogenesis (Shin *et al.* 1993). To validate EGFR expression as a biomarker useful as an intemediate end point, we are currently exploring this biomarker in a large number of samples in chemoprevention trials at M.D. Anderson Cancer Center.

Int-2/Hst-1 genes

After the *ras* gene family, the hst-1 gene is one of the frequently detected transformating genes (Sakamoto *et al.* 1986; Yoshida *et al.* 1988). Because this gene encodes a protein that is homologous to a fibroblast growth factor (FGF) and int-2-encoded protein, it is assumed to be a new member of the gene family that is involved in cell growth (Taira *et al.* 1987; Yoshida *et al.* 1987). Both hst-1 and int-2 genes are mapped to chromosome 11q13 (Casey *et al.* 1989), and their amplification has been reported in bladder (Tsutsumi *et al.* 1988), esophageal (Tsuda *et al.* 1989), melanoloma (Adelaide *et al.* 1988), and breast (Ali *et al.* 1989), and gastric (Yoshida *et al.* 1988). Co-amplfication of hst-1 and int-2 genes in a hepatocellular carcinoma was also accompanied by amplification of integrated hepatitis B virus DNA (Hatada *et al.* 1988). The biological significance of this coamplfication is presently not clear. The int-2 genes was amplified three to fivefold in 5 of 10 laryngeal carcinomas and two to threefold in 5 of 11 non-laryngeal carcnomas of the head and neck (Somers *et al.* 1990). In another report, int-2 was found to be amplfied in two of eight head and neck carcinomas (Zhou *et al.* 1988). There was a suggestion that amplification of int-2 was proportionally correlated with tumor recurrence and progression of clinical desease (Somers *et al.* 1990); a large patient population will be required to determine more precisely the significance of this gene amplification in head and neck carcinomas.

To localize the amplified region on the chromosome and to determine when during tumorigenesis this amplification occurred, we examined head and neck squamous cancer cell lines and their original paraffin-embedded tissue sections by ISH using both a cosmid probe for the int-2 gene and a biotin-labeled chromosome 11 painting probe. Three of ten cell slines exhibit-ed int-2 amplification, of which two (cell lines 1386, and 1986) were on chromosome 11 distal to the single copy gene, and the third (886) on another chromosome. These findings correlated well with the amplification of int-2 by Southern blotting. Paraffin blocks of those original tumors that contained adjacent premalignant lesions were analyzed with the int-2 probe to allow visualization of the timing of amplification. The original tissue of cell line 1386 and 1986 showed int-2 amplification in dysplastic and cancerous regions, while the third (cell line 886) showed amplification in the hyperplastic and dysplastic areas, carcinoma in situ and tumor areas. These results suggest that int-2 amplification may be used as a marker assess to cancer risk in future chemopevention trials.

p53 tumor suppressor gene

The *p53* gene, where encodes unclei protein, has been mapped to the short arm of chromosome 17 (17P13). This gene was originally identified as a nuclear protein that binds to the large T-antigen of the SV40 DNA tumor virus (Lane *et al.* 1970; Linzer *et al.* 1979). Although the *p53* gene was initially thought to act as a dominant oncogene, further investigation indicated that a mutant for of *p53* existed (Finlay *et al.* 1989). When the wild type *p53* gene was tested for its ability to transform cells, it was discovered that this type of *p53* could suppress transformation,

whereas a mutant form of *p53* could induce transformation (Finlay *et al.* 1989). Many different types of alterations (rearrangement, deletion, insertion, or point mutation) occurring at different locations within the *p53* gene have been observed in a wide variety of cell lines and heman tumors (Baker *et al.* 1990; Takahashi *et al.* 1991; Sidransky *et al.* 1991; Farrell *et al.* 1991; Bressac *et al.* 1991; Hsu *et al.* 1991; Chiba *et al.* 1990; Hollstein *et al.* 1991; Okamoto *et al.* 1991; cote *et al.* 1991; Bennett *et al.* 1991; Tamura *et al.* 1991; Osborne *et al.* 1991; Kovach *et al.* 1991; Shirasawa *et al.* 1991). The common genetic alteration found in the *p53* gene is a point mutation. Point mutation analyses have been confined primarily to exons 5 to 8, where the mutations are frequently found in phylogenetically conserved regions. Interestingly, however, the specific *p53* mutation site may reflect the different carcinogenic background of each tumors (Ishirka *et al.* 1991; Shaw *et al.* 1991; Brash *et al.* 1991).

It has been proposed that *p53* gene products function in cell cycle control (Kastan *et al.* 1992; Yin *et al.* 1992; Livingston *et al.* 1992). At least two stages in the cell cycle are regulated in response to DNA damage: G1-S and G2-M transitions. These transitions serve as checkpoints at which cell-cycle progression is delayed to allow repair of DNA damage before entering either the S phase, when damage would be perpetuated, or the M phase, when chromosome breaks would cause the loss of genetic material. Checkpoints are believed to be surveillance mechanisms that can detect DNA damage and active signal transduction pathways and then regulate replication or segregation machinery and possibly repair activity (Hartwell *et al.* 1989). Since there are mutants that abolish the arrest or delay that occurs in wild type *p53* in response to DNA damage, both G1-S and G2-M checkpoints are known to be under genetic control. Strong evidence exists that *p53* is necessary for the G1-S transition because high levels of *p53* inhibit cell cycle progression at the G1-S checkpoint (Kastan *et al.* 1992). Tumors cells lacking *p53* or having the dominant mutant form of it also lack the G1-S delay that occurs upon exposure to ionizing radiation (Kastan *et al.* 1991). The loss of the G1 checkpoint in mammalian cells is not associated with increased sensitivity to the lethal effects of ionizing radiation (Slichenmyer *et al.* 1993). The G2-M checkpoint is abolished by the mutation of *p53* in a number of yeast genes (Rowley *et al.* 1992) and by the treatment of mammalian cells with caffeine (Busse *et al.* 1978). Also, epithelium of the head and neck area is constantly exposed to carcinogens, and *p53* may affect how these damaged cells respond to this insult.

Since normal *p53* protein has a very short half life (6~20 minutes), but the mutant form has a half life of up to 6 hours (probably as a result of the protein stabilization, which enables its detection by immunohistochemical methods), it may be inferred that expression of the *p53* protein is synonymous with mutation (Lane *et al.* 1990). Iggo *et al.* (1990) found increased *p53* oncoprotein staining in lung cancer samples from smokers. They reported elevated *p53* protein levels in 14 of 17 (82%) squamous carcinomas while only eight of 21 (38%) had *p53* expression in non squamous cell carcinomas. Similarly, Chiba *et al.* (1990) reported that 65% of lung squamous cell carcinomas had *p53* mutations compared with 36% of non-squamous cell carci-nomas. The association of smoking with squa-mous cell carcinomas of the lung provides fur-ther evidence for a link between *p53* mutations and smoking. In a similar study of *p53* in SCLC cell lines, D Amico *et al.* (1991) found that 100% of SCLC cells had *p53* mutations.

More recently, mutations in the *p53* gene have been identified in a truly preneoplastic lesion, Barrett's esophagus (Cason *et al.* 1991), a precursor to adenocarcinoma of the exophagus. One study reported that in bronchial epithelium, *p53* protein was detected in 0% of normal mucosa, 8.3% of squamous metaplasia, 37% of mild dysplasia, 94% of severe dysplasia, and 55% of carcinoma *in situ* lesions (Bennet *et al.* 1993). Another study (Nees *et al.* 1993) showed *p53* mutations in the respiratory epithelium either adjacent to or at a significant distance from primary head and neck tumors. These investigators also observed *p53* mutations at both locaions in the same patient and concluded that *p53* mutation is an early event in head and neck carcinogenesis; this also support the field cancerization hypothesis. A similar observation was made by Boyle *et al*

(1993). In our laboratory, we studied *p53* protein expression in the head and neck squamous cell carcinomas of 33 patients whose tissue sections also contained adjacent normal epithelium, hyperplasia, and/or dysplasia. Fifteen (45%) of 33 head and neck tumors expressed *p53* but none of the normal control tissue (from cancer free nonsmokers) expressed it. However, five (21 %) of 24 tissue samples of normal epithelium adjacent to tumors, 7 (29%) of 24 hyperplasia tissue samples, and nine (45%) of 20 dysplasia tissue samples expressed *p53* protein (Shin *et al.* 1994). We conclude that *p53* expression can be altered in the very early phases of head and neck tumorigenesis, even in histologically normal epithelium. Thus, *p53* expression may be an excellent biomarker candidate assessing cancer risk and may serve as an intermediate end point in chemoprevention trials (Shin *et al.* 1994).

Cell Proliferation markers

Another candidate for biomarkers will be a series of cell proliferation markers such as proliferating cell nuclear antigen (PCNA), Ki-67, and DNA polymerase-α PCNA is a 36-kD acidic nuclear protein whose expression is associated with the late G1 and S phases of the cell cycle (Celis *et al.* 1985). It is an auxiliary protein to DNA polymerase δ and plays a critical role in the initiation in cell proliferation (Bravo *et al.* 1980); the protein, a 261-amino acid polypeptide with high aspartic and glutamic acid contents, has been characterized previously (Bravo *et al.* 1987).

To understand better the relationship between PCNA expression in tissues and cell proliferation status, paraffin-embedded tissues from head and neck and correctal cancers were examined after patients received BrdU infusions (Lee *et al.* 1992). Adjacent tumor sections were analyzed for PCNA expression and for incorporated BrdU (using anti-PCNA antibody and anti BrdU antibody, respectively). Regions of tumors high in PCNA-positive cells also had high BrdU uptake and vice versa. In all cases, the proportion of PCNA-positive cells was higher than the proportion of BrdU-positive cells. These results were not surprising since PCNA is expressed in most cell proliferating phases in G1, S, and G2, whereas BrdU only marks the cells in S phase (Bravo *et al.* 1987). To see whether PCNA expression is dysregulated in tumors, 107 NSCLC tissue sections were examined for PCNA expression. Squamous cell carcinomas showed the heghest proliferative activity, with a mean of 40 % PCNA-positive cells (range, 2~90%). The PCNA-positive fraction became progressively higher in areas of squamous metaplasia and carcinoma *in situ* (Lee *et al.* 1990).

To understand better tumorigenesis in head and neck cancer, we studied 33 formalin-fixed, paraffin-embedded tissue specimens from five different sites of head and neck squamous cell carcinomas that contained adjacent normal epithelia, hyperplasia, and/or dysplasia (Shin *et al.* 1993). PCNA expression was assessed by semiquantitative scoring in three epithelial layers (basal, parabasal, and superficial). The labeling index (the total number of positively stained cells divided by the total number of cells counted) and the weight mean index of PCNA expression (the sum of the number of counted cells mutiplied by the degree of intensity (0 to 3) of each cell and divided by the total number of counted cells) were calculated to demonstrate the degree of PCNA expression. Interestingly, normal epithelium adjacent to the tumor had much more proliferative activity than did control epithelium (from cancer-free nonsmokers). Furthermore, PCNA expression increased as tissues progressed from normal epithelium to hyperplasia to dysplasia and then to squamous cell carcinomas ($p<0.001$); the total increase in PCNA expression ranged from four fold in adjacent normal epithelium to tenfold in squamous cell carcinomas (Shin *et al.* 1993). As tissue progressed to carcinoma, we observed not only increases in the number of proliferating cells but also in the amount of PCNA expression per labeled cell. We therefore conclude that PCNA expression could be a useful biomarker to assess the risk for and development of head and neck cancer and to serve as an intermediate end point in chemoprevention trials (Shin *et al.* 1993).

As part of an ongoing chemoprevention trial in chronic smokers (>15 pack-year smoking), we screened patients for squamous cell metaplasia and randomized them to receive either 13-cis retinoic acid or a placebo. Our group examined PCNA expression in bronchial biopsy sections

obtained form six standardized sites at the major bronchial trees (Lee *et al.* 1992) and evaluated 165 samples for PCNA expression and histologic status. Among 81 biopsy specimens that showed histologically normal epithelium, only 12 % had more than 1% PCNA-positive cells. In contrast, 37% (19 of 52) of hyperplasia samples and 50% (10 of 20) of metaplasia samples had more than 1% PCNA positive cells. Of samples having dysplastic histology, 58% (7 of 12 specimens) had more than 1% PCNA positive cells. These results indicate significant collation between the increase in cell proliferative activity and histologic progression in epithelium at high risk of tumor development. Yang *et al.* (1987) studied esophageal premalignant lesions and showed that the patterns of expression of the cell proliferation marker and tritiated thymidine incorporation were similar to the patterns of PCNA expression we observed in lung and head and neck cancers. Lipkin *et al.* (1988; 1989) reported similar findings in colon cancer. These two previous studies also suggested that chemopreventve drugs can suppress PCNA expression. A large-scale chemopreventive study employing biomarkers for oral premalignancies and second primary cancer is ongoing at M.D. Anderson Cancer Center. We hope to have obtain definite answers from this study in the near future.

Squamous cell differentiation markers

Upper aerodigestive tract epithelia differentiate along the squamous pathway in carcinogenesis, and many studies showed that retinoids inhibit this abnormal cell differentiation *in vitro* and *in vivo* (Lotan *et al.* 1980; Lippman *et al.* 1989). Before we studied these differentiation markers in clinical trials with humans, we used these markers, including cytokeratins, EGFR, and transglutaminase 1, in a 7, 12-dimethylbenz (a)anthracine (DMBA) induced hamster buccal pouch model (Shin *et al.* 1990). Three different cytokeratins including K14 (M, 55,000), K1 (M, 47,000) were assayed in this model. The normal hamster cheek pouch epithelium expressed K14 in the basal layer and the K13 in the suprabasal layer, whereas K1 was detected in hyperplasia. K14 was no longer restricted to the basal layer but was expressed in differentiated cells. The same pattern was observed in dysplasia and in squamous cell carcinomas. However, during all stages of carcinogenesis, K13 was preserved in hyperplastic epithelium including anaplastic or differentiated areas (Gimenez-Conti *et al.* 1990). In contrast, K1 expression began as weak and patchy after 2 weeks of DMBA treatment, but became stronger and more homogeneous by 8 weeks of treatment. K1 expression, however, was almost entirely absent in squamous cell carcinomas. Therefore, we conclude that the pattern of keratin expression could be an important tool of study of carcinogenesis (Gimenez-Conti *et al.* 1990).

Another marker for squamous cell differentiation is involucrin, one of the major proteins components of cornified envelopes (Eckert *et al.* 1989). This protein undergoes extensive crosslinking by the membrane-associated type 1 transglutanminase (TGase 1), which catalyzes the formation of ε-(r-glutamyl isopeptide) linkages between protein-bound glutamine residues and primary amines such as protein-bound lysine (Simon *et al.* 1985; Thacher *et al.* 1985). These proteins are expressed in the upper corneal layers of the epidermis (Thacher *et al.* 1985). Squamous differentiation of keratinocyes is usually accompanied by increases in levels of involucrin and TGase 1, but the expression of involucrin precedes that of TGase 1 (Simon *et al.* 1985). Involucrin is expressed in premalignant lesions and squamous cell carcinomas (Kaplan *et al.* 1984; Murphy *et al.* 1984), and TGase 1 is also expressed in benign and malignant neoplasms of the skin and in a DMBA-induced hamster model. These squamous cell differentiation markers were shown to be modulated by retinoid acid in cell lines (Ta *et al.* 1990; Rubin *et al.* 1986; Lotan *et al.* 1987). Perhaps this modulation by retinoic acids could be mediated through the nucleic retinoic acid receptors (RARs)m since RAR-β mRAN was strongly detected in normal and hyperplastic epithelium and since levels gradually decreased as the tissues progressed to head and neck carcinomas (Xu *et al.* 1994). The mechanisms of differentiation of squamous cells and their modulation by retinoids seem very complex and need fruther exploration in well-designed chemopreventions trials.

Biomarkers in chemoprevention trials

The goal of clinical trials of chemopreventive agents for upper aerodigestive tract cancers is to reduce the incidence of these cancers. As described previously, the major obstacle in such trials is that the study end point (i.e., cancer development) is distant that mandates long-term follow-up and is thus very costly. It would be ideal to identify an earlier intermediate end points in the study that could indicate whether chemopreventive agents are having an effect of the tissues at risk (Lippman *et al.* 1990; Schatzkin *et al.* 1990) and, if so, could reflect the mechanisms of these agents activity at the tissue level. The use of biomarkers for this intermediate end point is desirable for several reasons. First, ideally, biomarkers can be useful for assessing the risk of tumor development in high-risk tissues and premalignant lesions. Second' these markers would allow a better understanding of the pathobiology of the clinical outcome of chemopreventive treatment. Third, as an intermediate end point in clinical trials, such markers would be good indicators to predict responsiveness to an agent before the final end point (i.e., cancer development) is reached. Thus, if one wanted to know whether the clinical outcome of treatment was the result of (a)reversal of the abnormal clones at the genetic level, (b)phenotypic reversal of the abnormal clones, (c)complete elimination of abnormal clones, or (d)partial suppression of less effective clones (if two or more distinct clones can be identified in the lesions), the answer could be derived by examining both multiple genetic and phenotypic markers on tissue samples obtained before and after chemopreventive treatment. Finally, although strict validation of any biomarkers as a true intemediate end point of cancer development may take many years of follow-up in a large-scale clinical trials, current biomarker candidates are an important adjuct to the development of new chemopreventive agents and to the rational design of future intervention trials.

We have described the candidates for genetic and phenotypic biomarkers, but we cannot overemphasize that these biomarkers must be validated in clinical trials. In the near future, validated comprehensive panels of biomarkers for early and intermediate stages of carcinogenesis may provide new standard end points and may even replace cancer incidence as the sole end point in chemoprevention trials.

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