Biomarkers in Chemoprevention for Upper Aerodigestive Tract Tumors

Dong Moon Shin, Jae Yoon Ro and Waun Ki Hong

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 nucleic antigen (PCNA) and the squamous cell differentiation 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
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), 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.

### 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

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<th>Table 1. Candidates of biomarkers for upper aerodigestive tract tumors</th>
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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 carcinogenesis 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 early 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 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 (Siebos 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 numbers 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 fit 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 aerodigestive tract cancer chemoprevention 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 lifestyle 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 tumorigogenesis 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 reported (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; Arnold et al. 1991; Hopman et al. 1991; Dhingra et al. 1992; Kim et al. 1993). This tech-
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Techniques allow 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 various 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 high-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 chromosom es 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 instability 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 mutations 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 triphosphate (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. 1982).
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 expression 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-dimethylbenz(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 code 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 intermediate 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 transforming 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 11ql3 (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-amplification 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 coamplification 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 carcinomas of the head and neck (Somers et al. 1990). In another report, int-2 was found to be amplified 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 disease (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 lines exhibited 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 chemoprevention 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 human 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 (Ishirika 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 carcinomas. The association of smoking with squamous cell carcinomas of the lung provides further 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 esophagus. 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 locations in the same patient and concluded that p53 mutation is an early event in head and neck carcinogenesis. This also supports 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 the relationship between PCNA expression in tissues and cell proliferation status, paraffin-embedded tissues from head and neck and rectal 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 highest 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 multiplied 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 chemopreventive 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)anthracene (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 cross-linking by the membrane-associated type 1 transglutaminase (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 keratinocytes 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 retinoid acids could be mediated through the nucleic retinoic acid receptors (RARs) in 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 further 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 point 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 intermediate end point of cancer development may take many years of follow-up in a large-scale clinical trials, current biomarker candidates are an important adjunct 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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