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Invited Review

Biomarkers of squamous cell carcinoma of the head and neck

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Summary. Despite the remarkable advances in cancer treatment, the 5-year survival rate for head and neck squamous cell carcinoma (HNSCC) has improved only marginally over the past 20 years. Investigators have attempted to develop new therapeutic methods to improve the survival rate from these tumors. Another approach, chemoprevention, has recently been tried to reduce cancer incidence. Biological understanding of tumorigenesis is critically important to provide risk assessment and intermediate end points during chemoprevention trials. Based on the fact that HNSCC develops through a multistep process by the accumulation of genetic and phenotypic changes in the field exposed to carcinogens, the investigation of specific biomarkers that represent each step must be valuable, since these biomarkers could be used to inhibit and/or to reverse the pathway of carcinogenesis. The potential biomarkers are classified as nonspecific and specific genetic biomarkers, proliferation markers, and differentiation markers in this review. Their usefulness in predicting tumors' biological behavior and responses to treatment and in monitoring the preventive effects of chemoprevention trials is also discussed.

Key words: Biomarkers, Squamous cell carcinoma, Head and neck, Prognosis, Chemoprevention

Nonspecific genetic markers

Micronuclei

Micronuclei have been widely used as an indicator of high cancer risk in fields exposed to carcinogens. Micronuclei are a reflection of clastogenic events and indicate the ongoing process of DNA damage (Prasad et al., 1995). A series of clinical trials has shown that high numbers of micronuclei correlate with a cancer risk in target tissues of individuals at high risk for the development of head and neck squamous cell carcinoma (HNSCC), such as smokers and betel quid chewers (Stich et al., 1988; Stich and Dunn, 1988; Benner et al., 1994). These investigators reported that treatment with beta-carotene, vitamin A, or alpha-tocopherol reduced the number of micronuclei in the target tissue. Although only a single marker of micronuclei is not sufficient to allow precise evaluation of the degree of overall genetic alterations, micronuclei studies may reveal the utility of screening the field exposed to carcinogens in assessing the risk of cancer development in target tissues (Shin et al., 1994a).

Chromosomal instability

Considering that HNSCC develops because of accumulated genetic alterations, the measurement of generalized genetic instability might be useful in assessing the risk of cancer development and monitoring the effects of chemopreventive agents. Genetic instability includes gene mutations, gene amplifications, chromosomal structural rearrangements and defects, and chromosomal aneuploidy (Boone et al., 1990). Among these genetic events, abnormal chromosomal numbers have been relatively easily detected by DNA flow cytometry (Landberg et al., 1990). But DNA flow cytometry has the disadvantages that it requires disruption of the cells' morphologic architecture and is not capable of selective analysis of tumor cells. The recently developed in situ hybridization (ISH) technique allows the detection of chromosomal abnormalities directly in interface cells (Hopman et al., 1986; Pinkel et al., 1986; Cremer et al., 1988; Van Dekken et al., 1990; Reid et al., 1992). Furthermore, ISH has been adopted for use on formalin-fixed, paraffin-embedded tissue sections by non-isotopically labeled 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 technique allows direct visualization of chromosomal abnormalities in malignant

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and premalignant tissues with preservation of morphologic architecture.

Voravud et al. (1993) performed ISH on chromosomes 7 and 17 in HNSCC tissues to correlate genetic instability with histologic changes. We found that there was a general trend toward increased genetic instability as the tissue progressed from normal through dysplasia to invasive carcinoma. Lee et al. (1993) demonstrated that increased polysomy (≥ 3 signals per cell) was associated with histologic progression from normal to dysplasia in oral leukoplakia. We have recently studied premalignant oral leukoplakia lesions with chromosome 9-specific probes. We observed higher polysomies in hyperplasia adjacent to dysplasia than in isolated hyperplasia. These results suggest that chromosome polysomies mark the biological characteristics beyond histologic property and help in cancer risk assessment. Taken together, these studies suggest that measurement of generalized chromosomal instability may provide a genetic marker for assessing the risk of cancer development in oral leukoplakia and for monitoring the effects of chemopreventive agents.

Oncogenes

Ras gene family

Human tumorigenesis is often described as a genetic disease because it involves both activation of oncogenes and inactivation of tumor suppressor genes (Chang et al., 1993). Activation of a proto-oncogene which controls cell growth and proliferation has been considered to play an essential role in dysregulating cellular proliferation (Bishop, 1987; Bos, 1988, 1989). The ras gene family is one of the most commonly altered proto-oncogenes in human cancers (Barbacid, 1981; Bos, 1988; Maubruck and McCormick, 1991). When transfected to NIH/3T3 murine fibroblasts, genes of the ras family can induce neoplastic transformation of the cells (Krontiris and Cooper, 1981; Perucho et al., 1981; Shih and Weinberg, 1982). Normal ras genes encode a 21-kDa protein (Brown et al., 1984; McGrath et al., 1984; Bos, 1988; Boguski and McCormick, 1993). The ras proteins are located on the inner side of the plasma membrane (Willingham et al., 1980; Chen et al., 1985; Buss and Sefton, 1986) and have guanine triphosphate (GTP) binding activity (Scolinick et al., 1979; Shih et al., 1980). The ras proteins also possess GTPase activity that eventually leads to oncogenic activation (Gibbs et al., 1984; McGrath et al., 1984; Manne et al., 1985). Mutation of codons 12, 13, or 61 induces loss of GTPase activity or binding activity of ras proteins (Bos et al., 1984; Verlaan-de Vries et al., 1986). The enhanced expression of ras proteins by amplification of the genes or insertion of a powerful promoter or enhancer can also activate ras genes (Pulciani et al., 1985; George et al., 1986; McKay et al., 1986).

Mutationally activated ras genes have been found in a wide range of human tumors. K-ras is particularly associated with adenocarcinomas and has been reported to be activated in pancreatic cancers (Almoquera et al., 1988; Smit et al., 1988; Renewald et al., 1989), colorectal cancer (Vogelstein et al., 1988; Bos et al., 1987; Forrester et al., 1987), and adenocarcinoma of the lung (Rodenhuis et al., 1988; Bos, 1989). Mutation of ras genes has also been reported in 27-56% of myeloid disorders (Janssen et al., 1987; Needleman et al., 1987; Farr et al., 1988b). Tumors of the skin, breast, and esophagus, non-Hodgkins' lymphomas, and SCC of the lung also showed ras gene mutations (Kraus et al., 1984; Spandidos, 1987; Hollstein et al., 1988; Leon et al., 1988; Neri et al., 1988: Rodenhuis et al., 1988; Bos, 1989; Rumsby et al., 1990; Van der Schroeff et al., 1990). In HNSCC, the incidence of mutation of ras genes is variable. A study done in India reported an incidence of 35% (Saranath et al., 1991), while an investigation in Europe reported that ras mutation in HNSCC was rare (Chang et al., 1991; Clark et al., 1993; Yeudal et al., 1993).

Sagae et al. (1989) reported that p21 overexpression was associated with poor prognosis in uterine cervix carcinoma. Pei et al. (1994) found that H-ras mutation was associated with metastasis in pituitary tumors. Azuma et al. (1987) reported that ras gene expression correlated with the degree of tumor differentiation and clinical stages in head and neck and lung cancers, while other investigators found no correlation between ras gene mutation and clinico-pathologic findings in lung and HNSCC (Sheng et al., 1990; Rodenhuis and Slebos, 1992; Scambia et al., 1994).

A few studies have been reported on ras gene mutations in premalignant lesions. In colonic adenomas from patients with familial polyposis coli, K-ras mutations were found in noncancerous adenomatous polyps, indicating that K-ras mutations could be a useful marker in colonic tumorigenesis (Farr et al., 1988a). A study of the expression of the H-ras gene was at different stages of experimentally induced oral squamous cell carcinomas demonstrated that H-ras was overexpressed at a very early stage of tumor development, suggesting that H-ras is a potential biomarker of tumorigenesis (Husain et al., 1989). Saranath et al. (1989), however, demonstrated the amplification of ras genes in advanced stage oral carcinomas. Yeudal et al. (1993) detected no ras gene mutations in oral premalignant lesions. Thus, further study of ras gene mutations is necessary to determine their usefulness as a biomarker for predicting prognosis or assessing the risk of tumor development.

Int-2/Hst-1 Genes

The Hst-1 gene was originally identified as a transforming gene in DNAs from human stomach cancers and a noncancerous portion of human stomach mucosa by transfection assay in mouse NIH/3T3 cells (Sakamoto et al., 1986). This gene encodes a protein that is homologous to a fibroblast growth factor (Yoshida et al., 1987). The Hst-1 gene mapped to human

chromosome 11 at band q13.3, a locus that is coamplified with int-2 in some human cancers (Yoshida et al., 1988; Casey et al., 1989). The coamplification of int-2 and hst-1 genes has been reported in bladder cancers (Tsutsumi et al., 1988), esophageal cancers (Tsuda et al., 1989), melanoma (Adelaide et al., 1988), breast cancers (Ali et al., 1989), and gastric cancers (Yoshida et al., 1988). Lese et al. (1995) reported the coamplification of int-2 and hst-1 genes in oral squamous cell carcinoma. Other investigators (Zhou et al., 1988; Somers et al., 1990; Yin et al., 1991) also found int-2 to be amplified in HNSCC. Somers et al. (1990) suggested that the amplification of int-2 correlated with tumor recurrence and progression of the disease.

To identify the region amplified, we studied HNSCC cell lines and the tissue sections from which they were established, and applied a cosmid probe for the int-2 gene, and a total chromosome 11 painting probe (Roh et al., 1994). One line did not show amplification, but three did, on chromosome 11 distal to the single copy 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 at the hyperplasia-to-dysplasia transition. Together, these results suggest that int-2 can be amplified in premalignant lesions before tumor development. Lese et al. (1995) reported that the amplification of these genes may be a late event during tumorigenesis, because they found no amplification of the genes in adjacent normal mucosa. Further study will be necessary to validate the usefulness of these genes as biomarkers for assessing the risk of cancer development in premalignant lesions.

PRAD-1 (CCND1) gene

PRAD-1 is located on 11q13 and was originally isolated as a gene overexpressed by juxtaposition to the parathyroid hormone gene on 11p15 in parathyroid adenosis (Rosenberg et al., 1991; Schuuring et al., 1992). It lies between the bcl-1 gene and hst-1/int-2 gene (Somers et al., 1990). PRAD-1 encodes cyclin D1, which is committed to controlling the cell cycle at the G1-S transition by interacting with the Rb gene product (Dowdy et al., 1993; Jiang et al., 1993; Williams et al., 1993) and by binding and activating cyclin-dependent kinases CDK-4 and CDK-6 (Rosenberg et al., 1991; Matsushine et al., 1992), 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 oncoprotein no longer require cyclin D1 expression to progress through the cell cycle (Bates et al., 1994). Cells that overexpress cyclin D1 proliferate abnormally, have a short G1 phase, and depend less on growth factors than cells that do not overexpress cyclin D1 (Quelle et al., 1993). 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 in transforming squamous cells, it may confer some growth advantage to cells already transformed (Hinds et al., 1994).

PRAD-1 is amplified and overexpressed in breast and squamous cell carcinomas (Somers et al., 1990) and amplified in approximately 30-50% of primary head and neck cancers (Williams et al., 1993; Callender et al., 1994). Its amplification has been correlated in recent studies with high cytologic grade, infiltrative growth pattern, hypopharyngeal site, and lymph node involvement in recent studies (Williams et al., 1993; Callender et al., 1994; Parise et al., 1994). Its overexpression has been associated with a more rapid and frequent recurrence of HNSCCs (Michalides et al., 1995), resulting in shortened disease-free and overall survival in patients with operable disease (Parise et al., 1994). 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 (Rosenberg et al., 1991; Kerlseder et al., 1994; Lebwohl et al., 1994).

Growth factors and growth factor receptors

erbB1 or epidermal growth factor receptor

The v-erbB1 gene was originally discovered in the avian erythroblastosis virus (Vennestrom et al., 1982). The corresponding proto-oncogene was found to encode epidermal growth factor receptor (EGFR) (Lin et al., 1984; Ullrich et al., 1984), a transmembrane receptor, well known to have intrinsic tyrosine kinase activity. The binding to growth factors causes the receptors to become phosphorylated on tyrosine (Watson et al., 1992). Therefore, quantitative and qualitative abnormalities of EGFR may be responsible for deranged keratinocyte proliferation and differentiation (Groves et al., 1992).

Many investigators have reported overexpression of EGFR in HNSCC and attempted to correlate overexpression of EGFR with prognosis of the tumors. To begin with, Mukaida et al. (1991) measured EGFR level in esophageal carcinomas by [^{125}I] EGF binding assay. They found that the survival rate of patients with high EGFR levels was significantly lower than that of patients with low EGFR levels. Itakura et al. (1994) also reported poor prognosis in EGFR-overexpressing esophageal cancers, suggesting that level of expression of EGFR could have prognostic significance. Dassonville et al. (1993) found that overexpression of EGFR was associated with shorter relapse-free survival in upper aerodigestive tract cancers. Miyaguchi et al. (1991, 1993) reported that overexpression of EGFR correlated with the rate of recurrence of glottic and maxillary sinus carcinomas. However, according to the studies of Furuta

et al. (1992) and Frank et al. (1993), there was no correlation between overexpression of EGFR and the prognosis of nasal, paranasal, and hypopharyngeal cancers. The prognostic significance of EGFR expression in HNSCC is till being debated.

Several investigators (Gutowski et al., 1991; Lorz et al., 1991; Yoneda et al., 1991) investigated the therapeutic potential of agents that interfere with the EGFR pathway, such as anti-EGFR antibodies in EGFR overexpressing human cancers. By examining mice bearing xenografts of EGFR-overexpressing SCC, Modjtahedi et al. (1994) found that the tumors regressed after treatment with the monoclonal antibody against EGFR. They concluded that treatment of the antireceptor antibodies might induce growth inhibition and terminal differentiation in EGFR overexpressing SCC.

We studied tumor tyrosine kinase activity associated with EGFR and localization of anti-EGFR monoclonal antibody RG83852 in patients with non-small cell lung cancer and head and neck cancer (Perez-Soler et al., 1994). Fifteen patients were treated with escalating doses of the monoclonal antibody by continuous infusion for 5 days and fresh biopsy specimens were taken 24 hours after therapy. Tumor EGFR saturation with RG83852 and tyrosine kinase activity were assessed after the administration of the antibody by comparing EGFR tyrosine kinase activity in a malignant lesion before and after therapy. Based on tyrosine kinase activity, EGFR saturation was observed at a high degree at higher doses of RG83852, and upregulation of EGFR itself was achieved by this therapy. We concluded from this study that treatment with the anti-EGFR monoclonal antibody may enhance EGFR tyrosine kinase activity and be useful in enhancing chemotherapeutic efficacy (Perez-Soler et al., 1994).

The expression of EGFR in premalignant lesions has been studied. Shirasuna et al. (1991) reported that expression of EFGR was increased as the tissue progressed from normal to invasive carcinomas. Grandis and Tweardy (1993) found increased production of transforming growth factor (TGF)-alpha and EGFR mRNAs in the histologically normal mucosa of patients at risk for a primary or secondary head and neck cancer, suggesting that these may serve as markers of malignant transformation and prevention therapy. We (Shin et al., 1994c), also reported that EFGR dysregulation happened in two steps in head and neck tumorigenesis namely the moderate upregulation of EGFR expression in normal mucosa adjacent to tumor and the further upregulation during the progression from epithelial dysplasia to invasive carcinomas. These results indicated that overexpression of EGFR might be a useful marker in assessing the risk of tumor development in chemoprevention trials.

Transforming Growth Factor-Alpha

Transforming growth factor-alpha has homology to EGF and binds to EGFR to induce epithelial proliferation (Deuel, 1987). However, the association of TGF-alpha with head and neck tumorigenesis has been little studied. Grandis and Tweardy (1993), on the basis of TGF-alpha expression in the histologically normal mucosa of patients at risk for a primary or secondary head and neck cancer, suggested that TGF-alpha may play a role in malignant transformation. Beenken et al. (1994) reported the expression of TGF-alpha in oral leukoplakia and noted that TGF-alpha expression decreased during treatment with retinoid, suggesting that TGF-alpha may serve as an intermediate end point in cancer chemoprevention trials.

Basic Fibroblast Growth Factor (FGF) and Receptors

Basic fibroblast growth factor (FGF) is involved in proliferation and differentiation of a wide range of cell types, and its expression is associated with angiogenic activity (Folkman and Klagsbrun, 1987). Basic FGF has been identified in numerous normal (Cordon-Cardo et al., 1990) and malignant tissues (Morrison et al., 1993; Nanus et al., 1993; Yamanaka et al., 1993). There have been a few reports of basic FGF expression in HNSCC. According to Schultz-Hector and Haghayegh (1993), cancer cells showing strong positive staining for basic FGF were discovered to have higher proliferative activity than cells negative for basic FGF. In addition, endothelial cell doubling time was significantly shorter in basic FGF-positive areas than in basic FGF-negative tumor cells. They concluded that basic FGF production may correlate to growth rate in HNSCC. Myoken et al. (1994) investigated the level of acidic and basic FGF in oral SCC. Their results indicated that squamous cells expressed high levels of endogenous acidic and basic FGF and suggested that FGF contributes to cancer cell growth. Janot et al. (1995) studied the levels of basic FGF and FGF receptors in HNSCC. Because, as they had already shown the level of basic FGF in HNSCC was the same or reduced relative to non-malignant adjacent mucosa, their results could not provide evidence that basic FGF plays an autocrine role in SCC. Instead, they found that HNSCC cell lines expressed FGF receptor form 2-IIIb, to which keratinocyte growth factor binds with a high affinity, eventually inducing the proliferation of keratinocytes. As yet, the role and biological significance of basic FGF and its receptors during tumorigenesis of HNSCC remains to be proven.

Tumor suppressor genes

p53 Gene

The p53 gene was originally discovered as the producer of a nuclear protein that binds to the large T antigen of the SV40 DNA tumor virus (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow et al., 1982). Although the p53 gene was initially considered an oncogene, inducing transformation of the cells (Eliyahu et al., 1984; Jenkins et al., 1984; Parada et al.,

1984), further investigation revealed that wild-type p53 could suppress neoplastic transformation (Finlay et al., 1989; Hinds et al., 1989). This gene has been mapped at the short arm of chromosome 17 (17p13) (Tominaga et al., 1992). The wild-type p53 protein has a very short half-life, 4-5 minutes (Thomas et al., 1983; Reich and Levine, 1984), while mutant forms which gain protein stability have half-life of 6 hours (Finlay et al., 1988; Iggo et al., 1990). The normal function of p53 protein is determined by its three-dimensional structure (Oren, 1992); thus, structural alteration by genetic mutations inhibits its normal function (Gannon et al., 1990; Unger et al., 1992). Such mutations are called "dominant" because their phenotype is manifested in the presence of the wild-type gene; as it inactivates the wild-type gene function, it is referred to as a "dominant negative" mutation (Herskowith, 1987).

The wild-type p53 protein is well known to regulate cellular proliferation (Braithwaite et al., 1987; Gannon and Lane, 1989; Bischoff et al., 1990; Oren, 1992) and differentiation (Kastan et al., 1991b) and to induce apoptosis of cells with damaged DNA; it is associated with DNA repair (Kastan et al., 1991a; Levine et al., 1991; Yonish-Rouach et al., 1991; Lowe et al., 1993; Libermann et al., 1995). These functions are derived from p53's transcriptional activity, modulating the expression of several genes (Vogelstein and Kinzier, 1992). Wild-type of p53 transcriptionally activates the production of the p21 protein encoded by the WAF1/CIP1 gene, which was discovered as a potent inhibitor of cyclin and cyclin-dependent kinase complexes (El-Deiry et al., 1993; Harper et al., 1993; Xlong et al., 1993). Wild-type p53 eventually induces G1 arrest of the cell cycle through transcriptional activation of p21 (El-Deiry et al., 1994; Li et al., 1994; Liu and Pelling, 1995).

Most p53 gene alterations in human cancers have been found in exons 5 to 9 (Hollstein et al., 1991; Levine et al., 1991) and have been observed in a widely variety of human tumors, such as cancers of the stomach (Yamada et al., 1991; Matozaki et al., 1992), breast (Chen et al., 1991; Davidoff et al., 1991; Coles et al., 1992), urinary bladder (Sidransky et al., 1991), liver (Oda et al., 1992; Hollstein et al., 1993), cervix (Crook et al., 1991; Fujita et al., 1992), and colon and rectum (Shaw et al., 1991; Peidano et al., 1993; Lazaris et al., 1995). Alterations of the p53 gene are very common in HNSCC (Boyle et al., 1993; Burns et al., 1993; Chung et al., 1993; Yin et al., 1993; Chang et al., 1994; Shin et al., 1994b). According to the study of Yin et al. (1993), abnormalities of the p53 gene in HNSCC include 17p13 deletion and amplification of the non-deleted allele as well as point mutations. A series of studies indicate that such p53 genetic events may play an active role in tumorigenesis in HNSCC.

Besides having a role in tumorigenesis, p53 is believed to play a significant role in anticancer treatment response, in that the wild-type of p53 protein is able to induce apoptotic death of cancer cells. The relationship between p53 mutation and prognosis is unclear, since conflicting results have been reported. Rosen et al. (1995) and Preudhomme et al. (1995) reported a lack of association between p53 mutation and prognosis in node-negative breast carcinoma, Burkitt's lymphoma, and acute lymphoblastic leukemia. On the other hand, in several reports examining node-negative breast cancer (Isola et al., 1992; Thor et al., 1992; Allred et al., 1993; Silvestrini et al., 1993; Gasparini et al., 1994) and hematologic malignancies (El Rouby et al., 1993; Orazi et al., 1993; Wattel et al., 1994), p53 was found to have prognostic significance. The prognostic significance of p53 expression in HNSCC is also controversial. Ahomadegbe et al. (1995) reported that p53 gene mutation was not related to metastatic dissemination and year survival rates in HNSCC. The study of Nylander et al. (1995) of the relationship between p53 expression and cell proliferation in HNSCC showed that p53 expression was not associated with proliferative activity of tumor cells and had no prognostic significance. In contrast, Girod et al. (1994) and Gorgoulis et al. (1995) reported that p53 expression was positively correlated with proliferation activity as the tissue progressed from normal through dysplasia to the invasive carcinoma of HNSCC.

To determine whether p53 protein expression has prognostic significance or is associated with patterns of treatment failure, we examined protein expression in 69 patients with HNSCC (Shin et al., 1996). In our best knowledge, this is the first paper to address p53 implication on patterns of treatment failure in head and neck cancer. We found detectable levels of p53 expression in 41 of 69 patients. Rates of overall survival, time to recurrence, time to second primary tumors, and time to any failure in the p53-expressing group were significantly lower or shorter than those in the p53-negative group. The rate of second primary tumor development was also significantly higher in the p53-positive group than in the p53-negative group. Thus, we concluded that p53 expression may be a valuable marker for identifying individuals at high risk of developing recurrences and second primary tumors, who may benefit from adjuvant therapy after definitive local therapy. The mutation of p53 genes has been discovered in premalignant lesions of gastric mucosa and Barrett's esophagus (Cason et al., 1991; Shiao et al., 1994). Many investigators (Warnakulasuriya and Johnson, 1992; Dolcetti et al., 1992; Field et al., 1993; Girod et al., 1994; Shin et al., 1994b; Gorgoulis et al., 1995) demonstrated the mutation and expression of p53 in premalignant lesions of HNSCC. These investigators concluded that p53 mutation is an early event in head and neck carcinogenesis, suggesting that p53 plays an active role in tumor progression. Shin et al., 1994d also studied chromosomal alteration (i.e., genomic instability) in association with p53 status in premalignant lesions and HNSCC and found that p53-expressing premalignant lesions (i.e., hyperplasia or dysplasia) showed higher chromosomal instability than p53-negative lesions, suggesting that altered p53 function enhances genomic instability during head and neck tumor progression. Therefore, p53 expression may

be an excellent biomarker for assessing the risk of cancer development and monitoring the effects of chemopreventive agents (Hong et al., 1990; Kelloff et al., 1994).

Chromosome 9p

van der Riet et al. (1994) discovered the loss of another tumor suppressor gene, chromosome 9p21-22, in head and neck cancers. They reported that the loss of this gene was lost in the majority (72%) of 29 cases of HNSCC. This gene was also lost in preinvasive lesions, suggesting that the loss of chromosome 9p is an early event in HNSCC, although the importance of this gene still remains unclear and further studies are needed.

Cell proliferation markers

Proliferating cell nuclear antigen (PCNA), an auxiliary protein of DNA polymerase delta, is known to be an excellent marker representing DNA synthetic phased cells (Bravo, 1986; Bravo et al., 1987; Dierenconck et al., 1991). The measurement of proliferating activity may allow prediction of the biological behavior of tumors and distinction of malignant from premalignant lesions (Robbins et al., 1987). Immunohistochemical techniques for cell cycle-related antigens has been extensively studied. PCNA is also known to be associated with DNA repair processes and may be expressed in cells not synthesizing DNA because of growth factor-mediated stabilization of PCNA mRNA and regulation of protein expression (Hall and Levison, 1989; McCormick and Hall, 1992). The Ki-67 antigen has been reported to be another marker of proliferating cells, maximally expressed during S phase (Brown and Gatter, 1990); it is rapidly degraded after mitosis (Bruno and Dazynkiewicz, 1992). The PCNA index is considered to be higher than the real value of the proliferating activity of the tissues, while the Ki-67 index is lower than the real value. DNA flow cytometry made it possible to secure information about the kinetics, especially ploidy, of the tumor cells (Landberg et al., 1990), although this method could not correlate DNA ploidy with the morphologic architecture. In addition, a technique involving administration of the thymidine analogue bromodeoxyuridine (BrdUrd) before biopsy, followed by staining with an anti-BrdUrd antibody, can be applied to obtain kinetic information about the tumor cells (Wilson et al., 1988). The nucleolar organizer regions (NORs) contain ribosomal genes and can be detected by silver staining (Howell, 1982). Because the regions visualized by AgNOR staining represent nucleoplasmic protein, such as RNA polymerase I, rather than NOR, AgNOR staining apparently evaluates the overall metabolic activity of the cells and not proliferation activity directly (Crocker et al., 1988).

An attempt to predict the prognosis through response to therapy by using the proliferating index has been made in HNSCC. Gunzl et al. (1993) measured PCNA index in patients with oral SCC, who received preoperative radiation therapy. They found that the patients in whom radiation therapy was successful showed higher PCNA levels than the patients with poor response. Jones et al. (1994) failed to correlate proliferating activity to any host or tumor factors in HNSCC. Neither PCNA nor Ki-67 values were significantly different between irradiated and nonirradiated tissues, patients who did and did not have lymph node metastasis, nor metastatic sites. In contrast, Tsuji et al. (1992a) reported that PCNA index decreased significantly after chemotherapy in oral cancers, suggesting that PCNA index can be useful to estimate response to anticancer agents.

The measurement of proliferating activity has been used to determine the grade of precancerous lesions in the tumorigenesis of HNSCC. Kobayashi et al. (1995) reported that proliferating index increased along with severity of epithelial dysplasia in the oral mucosa. Tsuji et al. (1992b) found that PCNA expression was significantly higher in premalignant lesions than in normal mucosa. Coltrera et al. (1992) observed that PCNA was expressed only in the basal layer in hyperplastic oral mucosa but extended to the suprabasal layers in severe dysplasia and carcinoma in situ. Shin et al.(1993) also performed immunohistochemical staining of PCNA in HNSCC found that normal epithelium adjacent to the tumor showed much more proliferation activity than the controls and that PCNA expression increased as the tissues progressed from adjacent normal through dysplasia to invasive carcinomas. Taken together, these studies indicate that PCNA could be a useful marker to differentiate normal tissue without cancer risk from normal tissue adjacent to cancer tissue and to determine the grade of epithelial dysplasia and that it may serve as an intermediate end point in chemopreventive trials.

Cell differentiation markers

The keratins, a family of water-insoluble proteins (40-70 KDa), can be divided into two groups, the relatively acidic and basic (Elchner et al., 1984), which are coordinately expressed in all kinds of epidermis. The expression of specific keratins may correlate with the type of epithelial differentiation (simple vs. stratified; keratinized vs. nonkeratinized) (Tseng et al., 1982; Cooper et al., 1985). Therefore, to study the ways that keratin expression is altered during HNSCC tumorigenesis may be valuable for gaining a better understanding of carcinogenesis and for developing an intermediate end point in chemoprevention trials (Gimenez-Conti et al., 1990).

Watanabe et al. (1995) investigated the alteration of cytokeratins and involucrin in SCC of the skin. The pattern of expression of cytokeratins in well-differentiated SCC was similar to that in normal epidermis, while the expression of other keratins decreased and altered in simple or nonkeratinized epithelium in poorly differentiated SCC. Watanabe et al. (1995) suggested that the change of simple or

nonkeratinized keratins in cutaneous SCC may be a marker of the cells' invasive and metastatic potential. In an experimental study of 7,12-dimethylbenz(α)-anthracene (DMBA)-induced SCC in hamster buccal pouch, Gimenez-Conti et al. (1990) observed the alteration of keratin expression as the tissue progressed from normal to invasive carcinoma. The normal hamster pouch expressed K14 in the basal layer and K13 in the suprabasal and differentiated cell layers and did not express K1. K14 expression extended to suprabasal and differentiated cell layers in DMBA-induced hyperplasia, dysplasia, and carcinoma. After DMBA treatment, K1 was expressed and its expression increased during progression to carcinoma although K1 almost disappeared in SCC. Gimenez-Conti et al. (1990) concluded that such alterations of the pattern of cytokeratins could be an excellent tool for the study of carcinogenesis and chemoprevention.

Lindberg and Rheinwald (1989) studied the expression of K19 in normal oral mucosa and pre-malignant lesions. In nonkeratinized mucosa, whether normal or benign hyperplastic, K19 was detected in the basal layer. On the other hand, there was no expression of K19 in keratinized mucosa. In moderate to severe dysplasia or carcinoma in situ, regardless of the keratinization types of the epithelium, K19 was expressed strongly in the basal and suprabasal cell layers. The investigators suggested that suprabasal expression of K19 was associated with premalignant change in oral mucosa. Cooper et al. (1993) and Schulz et al. (1992) also reported that K19 could be a potential intermediate endpoint for chemoprevention trials in HNSCC.

Involucrin, the precursor protein of mature keratinocytes, is synthesized as a soluble protein in the suprabasal layer of stratified squamous epithelium (Eckert, 1989). Involucrin is expressed in the superbasal and differentiated superficial cell layers in the normal epithelium (Kaplan et al., 1984; Murphy et al., 1984; Eckert, 1989). Involucrin was reported to be expressed irregularly through the entire layer in the premalignant lesions and SCC (Kaplan et al., 1984; Murphy et al., 1984). Vigneswaran et al. (1989) demonstrated that the expression of involucrin was altered inconsistently as the tissue progressed from normal though dysplasia to invasive carcinoma; therefore involucrin may not be an appropriate marker to define disease progression in oral leukoplakia.

Retinoids are well known to regulate the morphogenesis, development, growth, and differentiation of the keratinocytes (Morris-Kay, 1992; Chambon, 1994). The effect of retinoids depends on two types of nuclear retinoid receptors, retinoic acid receptors (RAR)-α,ß,γ and retinoid X receptors (RXR)-α,ß,γ (Chambon, 1994). Xu et al. (1994) investigated the expression pattern of RARs and RXRs in HNSCC tumorigenesis. They found that the expression of RAR-ß was significantly and gradually lost as the tissue progressed from normal through dysplasia to invasive carcinoma (Xu et al., 1994; Lotan et al., 1995). This alteration of RAR-ß expression can be restored by treatment with isotretinoin (Lotan et al., 1995). They concluded that RAR-ß may have a role in mediating the response to retinoids and may be useful as an intermediate biomarker for chemoprevention trials in HNSCC.

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