Molecular pathology of head and neck cancer

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Summary. Squamous cell carcinoma of the head and neck region (HNSCC) is the sixth most frequent cancer worldwide, comprising almost 50% of all malignancies in some developing nations. In the United States, 30,000 new cases and 8,000 deaths are reported each year. Survival rates vary depending on tobacco and alcohol consumption, age, gender, ethnic background, and geographic area. This variability reflects the multifactorial pathogenesis of the disease. Early detection and diagnosis has increased survival but the overall 5 year rate of 50% is among the lowest of the major cancers. Differences between normal epithelium and cancer cells of the upper aerodigestive tract arise from specific alterations in genes controlling DNA repair, proliferation, immortalization, apoptosis, invasion, and angiogenesis. These proteins include both tumor suppressors and activating oncogenes which regulate a wide variety of intracellular signaling pathways. Included in these pathways are growth factor receptors, signal transducers, and transcription factors which regulate DNA damage response, cell cycle arrest, and programmed cell death. In head and neck cancer, alterations of three signaling pathways occur with sufficient frequency and produce such dramatic phenotypic changes as to be considered the critical transforming events of the disease. These changes include mutation of the p53 tumor suppressor, inactivation of the cyclin dependent kinase inhibitor p16, and overexpression of epidermal growth factor receptor (EGFR). This review will focus on the molecular changes which occur in these pathways and how they contribute to the pathogenesis of HNSCC.

Key words: p53, Epidermal growth factor receptor, p16, Cell cycle, Tumor suppressor

Introduction

Squamous cell carcinoma, the most frequent cancer of the head and neck region, is a disease caused primarily by tobacco carcinogens (Boyle et al., 1993). There is no known inherited form of the disease. Head and neck squamous cell carcinoma (HNSCC) has been shown to progress through a series of dysplastic histopathologic changes before becoming an invasive cancer. Multiple discrete HNSCCs may arise in the same individual either at the time of diagnosis or subsequently. Some HNSCCs are readily diagnosed in the clinical setting by visual and physical examination similar to that for skin cancer. The differences between normal epithelium of the upper aerodigestive tract and cancer cells arising from that tissue are the result of mutations in specific genes and alteration of their expression (for review see Brachman, 1994). These genes control DNA repair, proliferation, immortalization, apoptosis, invasion, and angiogenesis. Both activating oncogenes and inactivation of tumor suppressor proteins are involved in these pathways. These signaling networks regulate the cellular phenotypes of DNA damage response, cell cycle arrest, and programmed cell death. For head and neck cancer, alterations of three signaling pathways occur with sufficient frequency and produce such dramatic phenotypic changes as to be considered the critical transforming events of the disease. These changes include mutation of the p53 tumor suppressor, overexpression of epidermal growth factor receptor (EGFR), and inactivation of the cyclin dependent kinase inhibitor p16. Other changes such as Rb mutation, ras activation, cyclin D amplification, and myc overexpression are less frequent in HNSCC and have been extensively reviewed elsewhere (Paulovich et al., 1997; Evan and Vousden, 2001). This review will focus on the molecular changes which contribute to the pathogenesis of HNSCC.

The p53 tumor suppressor

The p53 gene is believed to be the most frequently
Mutated tumor suppressor in human cancer (for review see Greenblatt et al., 1994). p53 was originally believed to be an oncogene following its discovery in SV40 large T antigen transformed cells (for review see Levine et al., 1991). p53 was present at relatively high levels in transformed cells while its expression was low in normal counterparts. p53 null keratinocytes possessing an activated ras oncogene proliferated at a higher rate than those expressing the tumor suppressor (Weinberg et al., 1994). Grafting of these cells into nude mice resulted in less differentiated, more aggressive tumors. These cells were also less responsive to the differentiating effects of calcium and the growth inhibitory effects of transforming growth factor β. Thus wild type p53 was shown to suppress transformation and block oncogenesis. In many tumors, both p53 alleles had been mutated or deleted, indicating loss of heterozygosity at this locus.

Mutations in the p53 gene have been linked to Li-Fraumeni syndrome, an inherited cluster of cancers including that of soft tissue, bone, breast, brain, and bladder (for review see Ko and Prives, 1996). Affected individuals develop tumors at an earlier age with loss of heterozygosity at the p53 locus. Heterozygous p53 mutation as the predisposing event for Li-Fraumeni syndrome was subsequently confirmed by DNA analysis of relatives of affected individuals. p53 null mutant mice are prone to development of a variety of neoplasms by 6 months of age (Donehower et al., 1992). These tumors were predominantly lymphomas but also included a number of sarcomas. This evidence strongly supported a role for p53 as a tumor suppressor gene.

The p53 gene was localized to chromosome 17p13 spanning some 20 kb, consisting of 11 exons, and encoding a 2.9 kb mRNA. The protein itself is composed of 393 amino acids, localized to the nucleus, and is expressed in all cells. p53 protein is phosphorylated on several serine residues which may affect its nuclear localization (Fig. 1). Most alterations in the p53 gene are missense mutations occurring in the highly conserved central region (Prives, 1994). p53 protein has an acidic amino terminus containing the transcriptional activation domain, the central region which is high in proline residues and imparts conformation to the protein, and a basic carboxyl terminus. This C terminus contains the non-specific DNA binding domain, oligomerization, and nuclear localization sequences. The sequence specific DNA binding domain is located in the central region. Wild type p53 has a short half life on the order of minutes while mutant forms of the protein can persist for several hours. This may explain why p53 expression is apparently elevated in some tumors, including head and neck cancer (Watling et al., 1992).

Wild type p53 induces growth arrest when overexpressed in a number of cancer cell lines (for review see Levine, 1997). Activation of wild type p53 has also been shown to promote differentiation and cell death (for review see Vousden, 2000). These properties have been used to prevent establishment of tumors in animal models of neoplasia (Clayman et al., 1995). p53 responds to DNA damage by arresting the cell cycle in G1 or G2 until repair can be completed. If DNA damage cannot be repaired, p53 may activate target genes resulting in apoptosis. Cells lacking p53 or expressing a mutated form of the protein fail to arrest in G1 following DNA damage. The ataxia telangiectasia (ATM) protein, the mutated form of which causes sensitivity to DNA damaging agents and cancer predisposition, phosphorylates the amino terminus of p53 in response to DNA damage (Banin et al., 1998). This phosphorylation enhances the activity of wild type p53.

Control of p53 activity has been the subject of intense investigation (for review see Giaccia and Kastan, 1998; Oren, 1999). In addition to phosphorylation, acetylation is an important p53 modification that regulates its activity (Sakaguchi et al., 1998). p53 is acetylated in vitro by the histone acetyltransferases p300 and PCAF on discrete lysine residues in the carboxyl terminus. Acetylation of these residues enhances p53 sequence specific DNA binding. Following radiation exposure, p53 is acetylated followed by phosphorylation of serine residues in its amino terminus. This amino terminal phosphorylation then specifically inhibited acetylation of the carboxyl terminus. p53 can be deacetylated by histone deacetylase 1 (HDAC1; Luo et al., 2000). This group identified a p53 interacting protein identical to metastasis associated protein 2 which reduced acetylation of the tumor suppressor. The protein also repressed transcriptional activation by p53 and modulated growth arrest and apoptosis. These recent studies have shown an important role for post-translational modification in regulating p53 function.

p53 interacts with a variety of oncoproteins, many of which are of viral origin such as SV40 large T antigen and human papillomavirus E6 protein. Binding of these factors has been shown to inactivate p53 function and paradoxically extends the half life of the tumor suppressor protein. SV40 large T antigen prevents p53 binding to DNA whereas the E6 interaction promotes its degradation by the ubiquitin dependent proteasome system. Regulation of ubiquitin dependent p53 proteolysis by the cellular protein mdm2 has been the subject of several recent studies. Mdm2 is a potent
inhibitor of p53 activity (Haupt et al., 1997). Mdm2 binds to the transcriptional activation domain of p53 and inhibits its ability to regulate target genes. This is at least in part due to mdm2 dependent inhibition of p53 acetylation by p300 (Kobet et al., 2000). This inhibition was dependent on the amino terminus of mdm2. The inhibition was specific for p53 since mdm2 did not affect histone or p73 acetylation. Mdm2 also induces rapid degradation of p53 by ubiquination and subsequent proteolysis (for review see Prives, 1998). Mutation of lysine residues in the p53 carboxyl terminus did not affect DNA binding or transactivation but interfered with mdm2 mediated degradation (Nakamura et al., 2000). These mutant p53 proteins were predominantly found in the cytoplasm which was not due to defects in nuclear import. These studies dramatically illustrate the multiple protein interactions that regulate p53 function.

Wild type p53 activates a number of target genes which regulate DNA damage repair, growth arrest, and apoptosis (for review see Agarwal et al., 1998). Among these are GADD45, p21/WAF1/Cip1, and bax respectively (Fig. 2). Some cancer cells expressing mutant p53 failed to arrest in G2 following DNA damage and entered mitosis (Bunz et al., 1998). If the p21 gene was disrupted, wild type p53 was unable to induce G2 arrest after DNA damage. These studies suggest that certain p53 target genes must be intact for proper functioning of the tumor suppressor. If DNA damage is severe, p53 may trigger programmed cell death via pro-apoptotic genes such as bax. Replication of damaged DNA may cause additional mutations which result in tumorigenesis or cancer progression.

Some tumor derived mutations in p53 can abrogate specific functions of the protein. The 175P mutation can induce growth arrest in certain cells but lacks the apoptotic function (Rowan et al., 1996). Cell cycle arrest due to overexpression of this mutant occurred primarily in G1 phase. The mutant protein also retained transcriptional activation function and the ability to induce target genes such as bax and p21. This led to characterization of a number of p53 mutant proteins with amino acid substitutions at position 175 (Ryan and Vousden, 1998). Some of these mutants demonstrated normal cell cycle arrest and apoptotic functions. Others, like the tumor derived 175P mutant, induced cell cycle arrest but were defective in apoptosis. A third class was defective in both activities. Some of the mutants were temperature sensitive for apoptosis but retained cell cycle arrest function. A subset of these p53 mutant proteins was defective in induction of bax and IGF-BP3 but demonstrated apoptotic function.

With regard to HNSCC, 79% of cancers and 36% of dysplastic lesions were shown to have p53 mutations (Kashiwazaki et al., 1997). Hyperplastic lesions were negative for p53 mutations in this study. A higher incidence of p53 mutations have been detected in invasive carcinomas (75%) than in non-invasive cancers (35%; el-Naggar et al., 1995). p53 mutations were not detected in normal mucosal cells. This study also detected sequential mutations of different exons which suggested accumulation of alterations during neoplastic transformation. Differences in codon mutations of the same exon between dysplastic lesions and cancers pointed to independent clonal development. A higher frequency (46%) of p53 mutations in dysplastic lesions has been detected by other studies (Qin et al., 1999). The incidence of p53 mutations correlated with degree of dysplasia with significantly higher numbers found in smokers. In agreement with these studies, dysplastic lesions in non-smokers infrequently contained p53 mutations (Lazarus et al., 1995). These results indicate that p53 mutation and inactivation is an early event in head and neck tumorigenesis.

**Epidermal growth factor receptor**

The epidermal growth factor receptor (EGFR) gene encodes a transmembrane receptor for EGF and transforming growth factor α (TGFα). Ligand binding to the extracellular domain induces receptor dimerization and activation of the cytoplasmic tyrosine kinase (for review see Schlessinger, 2000). Many epithelial cancers including that of the head and neck overexpress EGFR, its ligands, or both (Eisbruch et al., 1987; Reiss et al., 1991). EGFR has been detected in the basal layer of normal oropharyngeal mucosa (Christensen, 1998). All cells from dysplastic head and neck lesions stained for EGFR as did the majority of carcinomas. Almost all cells in poorly differentiated head and neck tumors were positive for the receptor. Amplification of the EGFR gene has been demonstrated in cultured cells and tissues (Yamamoto et al., 1986; Eisbruch et al., 1987). EGFR overexpression may result in constitutive activity of the kinase domain and consequently increase downstream signaling such as that of the mitogen activated protein kinase pathway. The tyrosine kinase activity of the receptor results in autophosphorylation and recruitment of intracellular signaling molecules (Fig. 3). Most tyrosine autophosphorylation sites are located in noncatalytic regions of the receptor. These sites function to bind Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains of a variety of signaling proteins. This recruitment provides a means of assembling the complexes required for receptor signaling (Pawson and Schlessinger, 1993). Proteins

![Fig. 2. Activation of target genes by p53. Expression of a number of genes is regulated by p53 activation. Among these are the cyclin dependent kinase inhibitor p21WAF1/Cip1 which can induce cell cycle arrest following DNA damage, GADD45 which is involved in DNA repair, and bax which promotes apoptotic cell death if DNA damage is severe.](image-url)
such as Grb2 and Shc which contain SH2 and SH3 domains mediate interactions with signal transduction proteins. Grb2 links a variety of receptors with the ras/mitogen activated protein kinase (MAPK) pathway. Grb2 interacts with phosphorylated receptors via its SH2 domain and recruits the guanine nucleotide exchange factor Sos to ras at the inner cell membrane (Pawson, 1995).

Receptor tyrosine kinases stimulate GTP binding to ras (Schlessinger, 2000). As stated above, Grb2 recruits Sos to the plasma membrane to produce exchange of GTP for GDP on ras. Membrane recruitment of Sos can also occur by binding of Grb2/Sos to Shc through its PTB domain (Margolis, 1999). In its active GTP bound state, ras interacts with many proteins such as raf and phosphatidylinositol 3-kinase to simulate downstream effectors such as MEK and ERK. These MAPKs are translocated to the nucleus where they activate a number of transcription factors which control cellular proliferation, migration, and differentiation (Hunter, 2000).

EGFR null keratinocytes have been shown to be unresponsive to a variety of ligands (Dlugosz et al., 1997). Expression of oncogenic ras in these cells was able to produce a potent mitogenic response. Oncogenic ras suppressed terminal differentiation markers in EGFR -/- keratinocytes which produced significantly smaller papillomas in mouse skin carcinogenesis models. These papillomas demonstrated significantly reduced S phase fractions in the basal layer but much higher bromodeoxyuridine incorporation suprabasally. A subsequent study demonstrated that EGFR was required to maintain the proliferating population in the basal layer of these papillomas (Hansen et al., 2000). Cycling cells from the basal layer migrated to the suprabasal compartment, underwent cell cycle arrest, and prematurely differentiated. These studies illustrate the central importance of EGFR in transducing mitogenic signals in epithelial cancer cells.

**The cyclin dependent Kinase inhibitor p16^INK4A**

The cyclin dependent kinase inhibitor p16^INK4A regulates the activity of cyclin D/cdk4 in the G1 phase of the cell cycle and is a known tumor suppressor (Hall and Peters, 1996). p16 specifically inhibits cyclin D dependent kinases and loss of its expression has the same functional consequences as cyclin D overexpression. p16 can arrest the cell cycle in G1 phase by inducing accumulation of hypophosphorylated Rb. Cells lacking functional Rb are resistant to p16 mediated growth suppression (Sherr and Roberts, 1999). Dominant negative cdk4 mutants have been shown to inhibit cyclin D dependent kinase activity by mechanisms separate from p16 (Jiang et al., 1998). p16 dissociated cyclin D/cdk4 complexes by release of bound p27Kip1 while dominant negative cdk4 associated with these proteins. In p16 overexpressing cells, p27 formed complexes with cyclin E/cdk2 whose activity was subsequently inhibited. Cells overexpressing cyclin E were resistant to p16 mediated growth arrest, suggesting that cyclin E inhibition as well as that of cyclin D is required for p16 mediated growth suppression.

Studies have shown that all four INK4 cdk inhibitors can associate with cdk4 and cdk6 in vitro (Parry et al., 1999), p21Cip1/WAF1 and p27Kip1 cdk inhibitors formed stable complexes with cdk6 but associated only transiently with cdk4. Overexpression of p21 stimulated the formation of cyclin D/cdk4/p21 complexes, suggesting that p21 may counteract the effects of INK4 proteins. However, only p16 could form stable complexes with cdk4 in proliferating cells. p16 overexpression inhibited cdk4 and cdk2 activity by
altering the composition of the complexes (McConnell et al., 1999). In cells overexpressing p16, this inhibitor was found primarily in association with cdk4 due to displacement of cyclin D and p27. In this case, cyclin D associated with cdk2 without affecting its interaction with p27. Displacement of p27 from cyclin D promoted its association with cyclin E/cdk2 resulting in inhibition of this complex.

Alterations, in particular homozygous deletions, of the p16 tumor suppressor are frequently detected in head and neck cancer (Olshan et al., 1997; Fig. 4). Homozygous deletions or mutations of the p16 gene were found in four of nine head and neck cancer cell lines (Zhang et al., 1994). Eleven of 68 primary tumors had missense or nonsense base changes. Overall, 44% of cell lines and 10% of primary tumors had some alteration of the p16 gene. A separate study found that 83% of head and neck cancers lacked p16 protein as determined by immunohistochemistry (Reed et al., 1996). The p16 gene in 67% of these tumors harbored homozygous deletions, 21% were methylated, 1 tumor displayed rearrangement, and 1 tumor had a frameshift mutation. These studies indicated that p16 mutation was a frequent event in head and neck carcinogenesis and this inactivation occurred by several distinct mechanisms.

Transfection studies have demonstrated that p16 was a potent growth suppressor of head and neck cancer cell lines (Liggett et al., 1996). This growth inhibition occurred regardless of the p16 status of the cell lines. p16 was unable to inhibit the growth of HeLa cells; however the p16ß protein, which contains a separate association with cyclin E/cdk2, was unable to inhibit the growth of HeLa cells; occurred regardless of the p16 status of the cell lines. This growth inhibition of p16ß was effective in blocking G1 to S phase progression of the cell cycle.

Future challenges

Understanding the fundamental molecular changes of HNSCC may lead to improvements in therapy for patients with this disease. The use of adenoviral vectors to restore p53 expression, demethylating agents to re-express p16, anti-EGFR immunotherapy, and small molecule kinase inhibitors are in various stages of testing and clinical trials. Until the efficacy of these therapies is proven and they enter the broader clinical setting, surgery and radiation remain the preferred treatment for primary HNSCC with chemotherapy reserved for more widespread disease. As with all cancer, early detection of HNSCC offers the best therapeutic outcomes and patient survival.

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References


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