Molecular introduction to head and neck cancer (HNSCC) carcinogenesis

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Summary Of all human cancers, HNSCC is the most distressing affecting pain, disfigurement, speech and the basic survival functions of breathing and swallowing. Mortality rates have not significantly changed in the last 40 years despite advances in radiotherapy and surgical treatment. Molecular markers are currently being identified that can determine prognosis preoperatively by routine tumour biopsy leading to improved management of HNSCC patients. The approach could help decide which early stage patient should have adjuvant neck dissection and radiotherapy, and whether later stage patients with operable lesions would benefit from resection and reconstructive surgery or adopt a conservative approach to patients with poor prognosis regardless of treatment. In the future, understanding these basic genetic changes in HNSCC would be important for the management of HNSCC.

Introduction

Oral cancer is one of the few cancer types where it is possible to obtain biopsies at all stages of the disease and study the genetic progression of tumorigenesis to metastasis. The pioneering work on the characterization of genetic alterations of colorectal cancer by Fearon and Vogelstein has become a paradigm for other human neoplasias (Diagram 1). It is now proposed that HNSCC follows a similar genetic progression in its development from premalignant lesions such as leukoplakia, dysplasia, erythroplakia and lichen planus. The precise nature of genetic alterations occurring at each step is still unclear but Califano et al. 1996 have described a preliminary HNSCC molecular progression model from benign to the invasive state with microsatellite analysis for allelic loss at 10 major chromosomal loci. The spectrum of chromosomal loss progressively increased at each histopathological step from benign hyperplasia to dysplasia to carcinoma in situ to invasive cancer (Diagram 2). Current established view on HNSCC formation requires the following three genetic alterations. The alterations are the p53 tumour suppressor protein, inactivation of the cyclin dependent kinase inhibitor p16 and overexpression of epidermal growth factor receptor (EGFR) in 40%, 70%, and 90% of all oral cancers, respectively. With the advent of cDNA profiling of many thousands of genes in a single assay, a wide range of
molecular markers are currently being identified that can distinguish patients' prognosis preoperatively. Traditionally, carcinogenesis and tumour progression has been attributed to alterations in oncogenes or tumour suppressor genes. This review will focus on the various established types of genetic alterations in HNSCC.

Hereditary

There is now increasing epidemiological evidence from case control studies of HNSCC patients that a family history of HNSCC is a risk factor. One matched case-control study using first-degree relatives of patients with new HNSCC, and first-degree relatives of the patients' spouses as controls, demonstrated an increased relative risk of 3.5 in association with a positive family history. Another large age and sex-matched study adjusting for alcohol consumption showed a similar relative risk of 3.65 in association with a positive family history.

The oncogenes

Oncogenes encode proteins that result in abnormal cell growth or tumorigenesis when overexpressed or mutated. The majority of these oncogenes are growth factors or growth factor receptors (hst-1, int-2, EGFR/erbB, c-erbB-2/Her-2, sis), intracellular signal transducers (ras, raf, stat-3), transcription factors (myc, fos, jun, c-myb), cell cycle regulators (Cyclin D1) and those involved in apoptosis (bcl-2, Bax). These growth regulatory pathways can be altered by gene mutation, chromosomal translocation, gene amplification or retroviral insertion. Activation of a proto-oncogene can result in either a qualitative or quantitative change in the oncoprotein.

**Growth factor and receptors**

EGFR (epidermal growth factor receptor) and its ligands have been studied extensively in HNSCC. The m-RNA for EGFR was elevated in 69-fold in 92% of tumours when compared with normal mucosa. Overexpression of c-erbB-2 (Her-2), which is an EGFR like oncogene located on chromosome 17, has been observed in 75% of HNSCC patients and correlated to shorter survival.

**Chromosome 11q13 amplification**

Fractional or entire DNA loss of chromosome 3p was found in 97% and amplifications of 11q13 region in 70% of primary HNSCC tumours. Amplification and rearrangements of the 11q13 region in primary HNSCC tumours has been reported in between 30-60% of cases using Southern hybridisation analysis. Amplification of the 11q13 region was correlated with high grade, late stage, aneuploid tumours, poor prognosis, recurrence and distant metastasis.

The oncogenes present in 11q13 include int-2 (FGF-3), hst-1 (FGF-4), Cyclin D1 (prad-1, bcl-1) and ems-1. The int-2 (FGF-3) gene is a member of the fibroblast growth factor family, is amplified in 50% of HNSCC patients.

**The cell cycle regulator genes**

Cyclin D1 gene regulates initiation of DNA synthesis and G1/S transition of cells. It was initially identified as the bcl-1 gene at chromosome 11q13, at the site of translocation t(11:14) (q13;q32) in B-cell malignancies. Amplification of Cyclin D1 gene was reported in 34% of HNSCC. The dysregulation of cyclin D1 expression has been
shown to occur early during the tumorigenesis process in HNSCC and enables subsequent cyclin D1 gene amplification. The overall 5-year survival of HNSCC patients with cyclin D1 amplification or with protein over-production was significantly lower than that of patients with low cyclin D levels \( (P < 0.0001) \). 

Transcription factors

Saranath et al. in 1989 studied 23 primary HNSCC and observed a 5- to 10-fold amplification of one or more of c-myc, N-myc, Ki-ras and N-ras oncogenes in 56% of the tumour tissue samples, with these oncogenes not being amplified in the peripheral blood cells of the same patients. L-myc and H-ras were not amplified in any of the samples. The oncogene amplifications seemed to be associated with advanced stages of squamous cell carcinomas, with the ras and myc family oncogenes being amplified in stages 3 and 4. Fredericks et al. (1999) found levels of c-myc mRNA and protein were rapidly and profoundly suppressed after infection of HNSCC with wild-type p53. Suppression of c-myc using antisense oligodeoxynucleotides (in the absence of p53) was sufficient to trigger apoptosis in similar HNSCC cell lines, raising the possibility that the reduction of c-myc may be involved in at least one of the cell death pathways mediated by p53. 

Tobacco usage is considered to be one of the causes of HNSCC. K-ras amplification, point mutation and loss of allele have been associated with tobacco-induced HNSCC. The association of tobacco and p53 in HNSCC will be discussed further below.

Intracellular transducers

Up-regulation of EGFR occurs early in squamous cell carcinogenesis and is critical for the loss of growth control in a variety of human cancers, including HNSCC. The Jak/Stat signalling pathway transmits signals from many cytokines and growth factor receptors to target genes in the nucleus. In HNSCC cells culture, EGFR stimulation initiates signaling via persistent activation of selective STAT proteins. Grandis et al. 2000 were first to provide evidence that constitutively activated Stat-3 is an early event in head and neck carcinogenesis that contributes to the loss of growth control by an anti-apoptotic mechanism.

Nagpal et al. 2002 studied the expression of Stat-3 in various stages and sites in 90 HNSCC patients. Stat-3 was positive in 82.2%, with one of eight premalignant lesions showed intermediate Stat-3 accumulation and none in normal tissues.

The Tumour suppressor genes and proteins

Tumour suppressor genes serve as transducers of negative growth signals. These genes are involved in cell cycle regulation including cell cycle arrest and apoptosis. Tumour suppressor genes can be altered in their functions by several mechanisms including point mutations, deletions or binding with cellular and viral proteins.

Alterations in the p53 gene expression

The p53 gene is located on the short arm of chromosome 17p13.1 and encodes a 53 kDa nuclear phosphoprotein that maintains genome stability. It regulates cell cycle progression, cellular differentiation, DNA repair and apoptosis. Mutations of
the p53 protein are the most frequent genetic alterations found in human malignancies.40

Usually, one of the p53 alleles is lost through a deletion, and the other is mutated. Thus, the tumour cells are, for all practical purposes, homozygous for the loss of the p53 normal locus.41 The point mutations of the p53 gene cluster in exons 5–9, the exons that are highly conserved among species. The mutant p53 protein has a much longer half-life in comparison to the wild-type protein, which causes accumulation of mutant p53 in cells and aids in detection.40,42

The p53 protein can also be studied with panels of monoclonal and polyclonal antibodies that recognise wild-type and/or mutant p53.43 Several groups have developed immunometric-type assays for measuring mutant p53. These assays are effective in quantifying mutated p53 in tumour cell line lysates and tumour tissue homogenates monoclonal and polyclonal antibodies that recognise wild-type and/or mutant p53.44 Tumour types with p53 mutations include breast, colon, stomach, bladder, and testicular, as well as sarcomas and melanomas.43 The pattern of p53 expression in breast cancers, as judged by immunohistochemistry, can be used for tumour sub-classification.45–47

All studies examining the value of p53 as a prognostic indicator in various cancers have concluded that tumours tend to be more aggressive when they are p53-protein positive or when the p53 gene is mutated. Many studies have examined the prognostic value of p53 in breast cancer. There is agreement that p53 gene mutations or p53 protein accumulation in the tumour is strongly associated with ER negative and PR negative tumours and shortened disease-free and overall survival.48

Mutation in the p53 gene in HNSCC is found to occur early49 and the incidence is reported to be 50–60% of all HNSCC.50,51 Mutant forms of p53 have been associated with poor prognosis in HNSCC.52 Over 60% of mutation in the p53 gene for HNSCC occurs between codons 238–248 within exon 7.53 Kropveld et al. sequenced the entire RNA and DNA of the entire p53 gene, exons 1–11 in 25 patients with HNSCC and found p53 gene alterations in almost 100% of the HNSCC cases studied.54

Smoking is a well known associated risk in the development of HNSCC. Several studies have shown smoking55 and tobacco chewing56 causes p53 alteration in the early stages of HNSCC development.57

The expression of p53 protein in primary HNSCC was significantly predictive of shorter survival because of its association with earlier development of both tumour recurrence and second primary tumours.58 In histopathologically negative surgical margins and cervical lymph nodes of patients with HNSCC detection of p53 mutation is associated with increased risk of local recurrence.59

A number of studies have demonstrated the association of human papillomavirus (HPV) and HNSCC.60–62 The oncogenic potential of HPV 16/18 may be due to the ability of its E6 oncoprotein to promote degradation of wild-type p53 protein.63

Tumour suppressor gene p16 (CDKN2A, MTS1-multiple tumour suppressor 1)

The p16 protein is a tumour suppressor protein of 156 amino acids; 16.5 kDa in size and the gene, called CDKN2A (cyclin-dependent kinase inhibitor 2A) is located at 9p21.64 It interacts strongly with CDK4 and CDK6 and inhibits its ability to interact with Cyclin D and function as a negative regulator of proliferation of normal cells.65 This effect is mediated through the retinoblastoma protein (Rb), another tumour suppressor.

The Rb protein is found in the nucleus of cells. When active (unphosphorylated), it sequesters cellular proteins required for cell replication. There are now more than 20 cellular proteins known to bind to Rb. The binding of p16 to CDK4/6 and forming complexes with Cyclin D prevents Rb phosphorylation (activates Rb) leading to the inhibition of the cell cycle G1/S transition. Absence of p16 would result in an inactivation of Rb protein, thereby allowing an uncontrolled stimulation of cell growth.66

The primary role of p53 and p16 tumour protein is to hold a damaged cell in G1 phase before it enters S phase, allowing it be repaired. This prevents the damaged cell from replicating and being carried through the cell cycle, which can cause cancer. If the damaged cell cannot be repaired, the tumour suppressor genes will then function in killing the cell in a process known as apoptosis.67

In HNSCC, alteration of the p16 protein occurs early68 and can be affected by HPV human papillomavirus.62 Overall, alteration of the p16 protein occurs in 70% of all HNSCC4 with somatic mutation of the gene (CDKN2A) occurring in 10% and homozygous deletion occurring in 50% of cases studied.69 Methylation of CDKN2A is another important mechanism causing inactivation of this gene in HNSCC.69,70

Regulation of apoptosis

Bax, Bcl-2, and p53 proteins are involved in the regulation of apoptosis and have been reported
to correlate with prognosis in several tumour types. Over-expression of Bcl-2 and loss of Bax expression is significantly associated with poor prognosis. High apoptosis was significantly associated with high Bax expression and highly differentiated tumours.71

Other tumour suppressor genes associated with HNSCC

The p15 gene has been designated MTS2 ‘multiple tumour suppressor 2’ (CDKN2B). It is deleted in 10–50% of HNSCC.72 It is thought that loss of 9p is an early event in the development of HNSCC.2 Loss of heterozygosity (LOH) at 9p21-22 was reported in 72% of 29 HNSCC cases studied.73

The p21 gene is mapped to chromosome 6p21. The p21 tumour suppressor protein was found to inactivate cyclin E-cdk-2 and cyclin D1, D2, D3-cdk-4 complexes components of the regulatory kinases that target pRB for phosphorylation.74,75

The p27 gene regulates cell cycle progression from G1 to S phase. The p27Kip1 is a member of the CIP/KIP family of cdk inhibitors that negatively regulates cyclin-cdk complexes. Reduced levels of p27Kip1 protein have been identified in a number of human cancers, and in some cases reduced p27Kip1 is associated with an increase in proliferative fraction. Jordan et al. 1998 observed that p27kip1 protein was significantly reduced in oral dysplasias and carcinomas compared with normal epithelium. In addition, there was a significant reduction in p27Kip1 protein between low- and high-grade dysplasias, suggesting that changes in p27Kip1 expression may be an early event in oral carcinogenesis.76

Angiogenesis

Tumour growth is associated with elevated cellular activities and increased blood supply is crucial for its continuing development. The process of angiogenesis is in itself a multi-step process that appears to be regulated by both stimulatory and inhibitory factors.77 Steps critical to successful neovascularization include degradation of the extracellular matrix, endothelial cell proliferation, migration and remodelling of extracellular matrix.

Angiogenesis has been linked to increased metastasis formation and decreased overall

Box 1.

Explanatory box

**Allele:** Alternative states of a gene e.g. colour of eyes.

**Cyclins:** A class of proteins that fluctuate in concentration at specific points during the cell cycle and that regulate the cycle by binding to a kinase.

**Gene amplification:** A cellular process characterised by the production of multiple copies of a particular gene or genes to amplify the phenotype that the gene confers on the cell.

**Loss of heterozygosity (LOH):** Mechanisms leading to loss of the remaining normal allele.

**Interleukins:** Regulatory proteins that are released by lymphocytes and act as intercellular mediators in the generation of an immune response.

**Oncogenes:** Encode proteins that result in abnormal cell growth or tumorigenesis when overexpressed or mutated.

**Point mutation:** A mutation that changes only one small area or one nucleotide in a gene.

**Telomerase:** Telomeres are repeated DNA sequences that protect the ends of chromosomes from being treated like a broken piece of DNA needing repair. Telomeres are also thought to be the ‘clock’ that regulates how many times an individual cell can divide. Telomeric sequences shorten each time the DNA replicates. When the telomeres reach a critically short length, the cell stops dividing and ages (senesces), which may cause or contribute to age-related diseases. In cancer, an enzyme called telomerase is reactivated and maintains the length of telomeres, allowing tumour cells to continue to proliferate.

**Tumour suppressor genes:** Encodes proteins that inhibit tumour growth, traditionally preventing the cell from completing the cell cycle if its DNA is damaged or the cell has suffered from other types of damage.
survival in patients with various tumours, including HNSCC. The angiogenesis initiating signals are exemplified by vascular endothelial factor (VEGF), interleukin-8 (IL-8) and fibroblast growth factors (FGF 1/2). The inhibitors of angiogenesis signals are thrombospondin-1, which binds to CD36, a transmembrane receptor on endothelial cells. Expression of VEGF family members VEGF-A and VEGF-C in HNSCC has been associated with increased risk of lymph node metastasis. Angiogenesis factors associated with HNSCC development has been used as a therapeutic targets and currently only used experimentally. Many of the investigated angiogenesis inhibitors demonstrated anti-tumour effects in preclinical and clinical trials. In a few cases, partial remission was observed and appears to be promising candidates for a clinical use in the therapy of HNSCC in the future (Box 1).

Understanding these basic molecular changes would be important for the clinical management of HNSCC in the future.

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