Review Article

Oral squamous cell carcinoma: Etiology, pathogenesis and prognostic value of genomic alterations

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Abstract

Tumours of the head and neck comprise an important group of neoplasia, the incidence of which is increasing in many parts of the world. This increase remains high, despite all the advances in modern medicine. This malignancy is more prevalent in the developing world and unfortunately, has not received satisfactory attention as the more prevalent cancers of the developed world, like lung, breast, or colon cancer. Recent advances in diagnosis and therapeutic techniques of these lesions have yielded novel molecular targets, uncovered signal pathway dominance and advanced early cancer detection. This review covers recent advances in our understanding of the etiology, molecular changes and the possible role that genomic and proteomic research might play in the diagnosis and effective cure of this modern-day scourge.

Key words: Oral cancer, apoptosis, telomerase activity, LCM.

More than 95% of the carcinomas of the oral cavity are of squamous cell type, in nature. They constitute a major health problem in developing countries, representing a leading cause of death. The survival index continues to be small (50%), as compared to the progress in diagnosis and treatment of other malignant tumors. According to World Health Organization, carcinoma of oral cavity in males in developing countries, is the sixth commonest cancer after lung, prostrate, colorectal, stomach and bladder cancer, while in females, it is the tenth commonest site of cancer after breast, colorectal, lung, stomach, uterus, cervix, ovary, bladder and liver.[1]

Since, the oral cavity is more accessible to complete examination, it could be used in early detection of precancerous and cancerous lesions. But either due to ignorance or inaccessibility of medical care, the disease gets detected in the later stages. Thus, there is a need for improvement in early detection of oral carcinomas, because in the initial stages, treatment is more effective and the morbidity is minimal. One method that is currently being investigated, utilizes *in-vivo* toluidine blue staining with oral brush biopsy, for diagnosis of premalignant lesions, enabling the clinician to choose the patients for scalpel biopsy.[2]

Most invasive oral carcinomas are preceded by a preinvasive stage, that may last for many years. Tumor progression in epithelia has been classified as normal, hyperplastic (non-dysplastic), dysplastic carcinoma *in situ and* invasive carcinoma.[3] The majority of the initial alterations of precancerous and cancerous oral lesions are not readily recognizable, on clinical or histopathological examination. The basic biology of initiation and progression of these tumors is still obscure.

Epidemiology and Etiology

Overall incidence and mortality attributed to oral squamous cell carcinoma (OSCC) is increasing, with current estimates of age-standardized incidence and mortality of 6.6/100,000 and 3.1/100,000 in men and

2.9/100,000 and 1.4/100,000 in women, respectively.[1] Recent studies confirm that oral cancer forms a large part of the cancer load in parts of India.[4] Tobacco and alcohol are the two most important known risk factors for the development of oral cancer.[5] Cofactors in OSCC include dietary factors, immunodeficiency and viral infections like HPV 16/18.

Risk factors for oral cancer

The risk factors include tobacco associated intra-oral carcinogens, which may play a synergistic role in oral tumorigenesis. From relative risk factors of alcohol and tobacco, it has been estimated, that 75% of all oral cancers are preventable. In the remaining 25% of patients who are not exposed to these substances, the cause/s of their tumors remains unknown.[6] The disproportionately higher incidence of carcinoma of the head-neck in relation to other malignancies in India, may be due to use of tobacco in various forms, consumption of alcohol, low socioeconomic condition related to poor hygiene, poor diet and rampant viral infections.[7]

Tobacco

Oral neoplasia has been associated with chewing of tobacco with betel quid (BQ) in India and other asian countries, whereas in western countries, cigarette smoking and heavy alcohol consumption are the main risk factors.[8] The international agency for research on cancer (IARC) confirmed that smoking of various forms of tobacco (e.g., bidis, pipes, cigars and cigarettes) is carcinogenic in humans.[9] Chewing of tobacco with BQ increases exposure to carcinogenic tobacco-specific nitrosamines (TSNA) and to nitrosamines derived from areca nut alkaloids. Furthermore, reactive oxygen species (ROS) implicated in multistage carcinogenesis, are also generated in substantial amounts in the oral cavity during chewing. Tobacco smoke pro-carcinogens such as benzo-[α]-pyrene, are metabolized by oxidizing enzymes, particularly cytochrome *p450*, some resulting in the production of reactive carcinogenic intermediates. Some studies link that cytochrome P450 family 1, subfamily A (CYP1A1) and CYP2E1 genotype, shows susceptibility to oral cancer, but others have failed to confirm this association.[10]

Betel Quid and Areca Nut

Betel chewing is reported to be the most important etiological factor in oral submucous fibrosis. The use of betel quid, containing both areca nut and tobacco, is associated with a much higher relative risk of oral cancer, between 8-15 times as compared to that of 1-4 times, associated with using the quid, without tobacco.[8] BQ chewing produces ROS that is detrimental to oral mucosa and can be directly involved in tumor initiation process, by inducing mutation, or by making the mucosa susceptible to BQ ingredients and environmental toxicants. Betel quid (BQ) chewing produces reactive oxygen species (ROS), that have multiple detrimental effects upon the oral mucosa. The production and release of ROS occurs under alkaline conditions during the autooxidation of areca nut (AN) polyphenols, in the BQ chewer's saliva.[11] The ROS can be directly involved in the tumour initiation process, by inducing genotoxicity and gene mutation, or by attacking the salivary proteins and oral mucosa, leading to structural change in the oral mucosa, that may facilitate the penetration by other BQ ingredients and environmental toxicants. The nitrosation of areca alkaloids to AN-specific nitrosamines occurs in the saliva of BQ chewers.[12] These AN-specific nitrosamines are mutagenic, genotoxic and capable of inducing tumours in animal models.[13]

Alcohol

Alcohol, acting both independently as well as synergistically with smoking, has been implicated in oral carcinogenesis.[14] More importantly, alcohol may act as a solvent and enhance the penetration of carcinogens into target tissues. Acetaldehyde, which is the alcohol metabolite, has been identified recently as a tumor promoter.[15,16]

Viruses

Another risk factor is human papillomavirus (HPV), which is also closely associated with benign and malignant oral lesions. This virus is detected in condylomas, focal epithelial hyperplasia, squamous cell papilloma and malignant oral lesions. HPV positivity is higher in tumors from the oral cavity (59%), pharynx (43%) and larynx (33%).[17] Among those, only a small fraction of HPV-infected lesions rarely proceed to malignant transformation, specially those with HPV subtypes 16,18.[18-21] Hence, these studies indicate that tumorigenic conversion requires the presence of other risk factors.

Diet

The importance of diet and nutrition in oral neoplasia has been indicated in several epidemiological studies.[22,23] Fruits and vegetables (high in vitamins A and C) are described as protective in oral neoplasia, whereas meat and red chilli powder are thought to be risk factors. Although the individual micronutrients

responsible have not been formally identified, vegetables and fruits that protect against oral cancer and precancer, are rich in b-carotene, vitamin C and vitamin E, with anti-oxidant properties. Iron deficiency, resulting in oral epithelial atrophy and the Plummer-Vinson (Patterson Brown Kelly) syndrome, is associated with cancer of upper air and food passages and dietary iron may play a protective role in maintaining the thickness of the epithelium.[24]

Family History of Head and Neck Squamous Cell Carcinoma (HNSCC)

Epidemiological evidence from case-control studies of HNSCC, indicates that a family history of head and neck cancer is a risk factor. The ability to repair DNA damaged by tobacco carcinogens, such as benzo-[α]-pyrene diol epoxide, is defective in some patients with head and neck cancer. Head and neck cancer patients show an increased susceptibility to chromosome damage by mutagens.[25]

Villaret *et al* used cDNA array and identified genes such as keratin 17 and 19, laminin-5, connexin-26 and VEGF as being differentially expressed in HNSCC tissues, with respect to normal tissue.[26]

Immune Deficiency

A defective immune response, as seen in a human immunodeficiency virus (HIV)-infected individual, may predispose to cancer. The commonest oral malignancy in HIV-infected patients is Kaposi's sarcoma[27] and the Human Herpes virus type 8 (HHV-8) has been implicated as the aetiological agent.[28] Lymphoma, mostly non-Hodgkin B cell lymphoma in HIV-infected individuals, or other immunosuppressed states, is commonly associated with Epstein-Barr virus and may occur in the head and neck. Oral squamous cell carcinomas of the lip are more common in transplant recipients receiving immunosuppressive therapy, but HIV infection does not predispose to intra-oral squamous cell carcinoma.[29]

Candida

Candida albicans can induce epithelial proliferation and can produce carcinogens from procarcinogens *in vitro*. Chronic hyperplastic candidosis presents as nodular or speckled-white mucosal plaques. They are potentially malignant oral epithelial lesions.[30]

Molecular Changes in Oral Cancer

Cancer occurs through multiple steps, each characterized by the sequential stimulation of additional genetic defects, followed by clonal expansion. The genetic alterations observed in head and neck cancer are mainly due to oncogene activation and tumor suppressor gene inactivation, leading to de—regulation of cell proliferation and death. These genetic alterations, include gene amplification and overexpression of oncogenes such as myc, erbB-2, Epidermal Growth Factor Receptor (EGFR), cyclin D1 and mutations, deletions and hypermethylation leading to *p16* and *p53* tumor suppressor gene inactivation.[31]

Tumor Suppressor Genes (TSGs) and Growth Regulators

Growth regulators and TSGs act as transducers of negative growth signals. Genetic alterations involving the tumor suppressor genes *p16* and *p53,* are frequently observed in head and neck tumors. Genetic abnormalities inactivating the *p16* gene might confer cell growth defects, contributing to the tumorigenic process. These genes are involved in cell cycle regulation, including cell cycle arrest and apoptosis. Alteration in both alleles of a gene is required for the loss of function. The TSG *p53*, is called as 'Guardian of the Genome', having a role in maintaining genomic stability, cell cycle progression, cellular differentiation, DNA repair and apoptosis.[32] A number of findings indicate that *p53* plays an important role in cell-cycle control (both G1/S and G2/M checkpoints) and in the induction of apoptosis. The gene can be inactivated by several mechanisms, including point mutations, deletions and binding with cellular and viral proteins. *p53* gene-inactivation via the above mentioned factors, has been demonstrated in squamous cell carcinoma. Due to its high catabolic rate, it is not usually possible to demonstrate *p53* protein in normal tissues using immunohistochemical procedures, whereas mutated *p53* exhibits a much lower catabolic rate and accumulates in the cells.

Genomic instability

The evaluation of the genomic stability can be done using techniques such as Loss of heterozygosity (LOH) screening and comparative genomic hybridization (CGH). Loss of heterozygosity of the *p53* allele has been reported in 20% of OSCCs, as well as in 22% of premalignant oral lesions.[33] The risk of progression from premalignancy to cancer is low, when no genetic changes were seen, intermediate, if there is genetic loss on the short arms of chromosomes 3 and 9 (3p and 9p) and high, if there is 3p and 9p loss accompanied by genetic loss on additional chromosome arms, including 4q, 8p, 11q, 13q and 17p. The LOH of the H-ras allele may encompass a tumor suppressor gene in the vicinity of the oncogene. Loss of chromosome 11

alleles has been reported in a number of tumors. These techniques can be used to identify and confirm both known and unknown alterations (deletions, mutations) in the genomes of a variety of human tumors. EGFR and its ligands have been studied extensively in OSCCs. Over expression of EGFR and Transforming growth Factor (TGF) has been found to be associated with a decrease in the disease-free and cause-specific survival rates.[33] TGF-m-RNA was found at 5 times higher levels in 96% of histologically normal tissues examined from patients with OSCCs and at 5 times higher levels in 87.5% of tumors when compared with normal mucosa.[34]

Role of deregulated apoptosis in the pathogenesis of oral cancer

The deregulation of apoptosis-related genes, aids in successful carcinogenesis. The relative contribution of apoptosis and proliferation to disease progression in the oral mucosa, was examined using terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) assay and Ki-67 staining. Further apoptosis related cell cycle regulators, namely Retinoblastoma (Rb), cyclin D1 and Fragile histidine triad gene (FHIT) were analyzed for Loss of heterozygosity (LOH), gene amplification and aberrant transcripts, respectively in oral cancer samples. Status of *p53*, bcl-2 and bax, members of the *p53* dependent apoptotic pathway, were evaluated in oral cancers/oral lesions by immunohistochemistry. Frequent overexpression of apoptosis regulators p53, bcl-2 and bax, was observed in oral cancers and in a subset of oral lesions. It was further revealed that there is overexpression of anti-apoptotic members of the bcl-2 family namely, bclxL and Mcl-1, in oral cancer cell lines. These studies thus indicate, that evasion of apoptosis via abnormal expression of bcl-2, bclxL, mcl-1 and *p53,* may contribute to oral cancer pathogenesis.[35]

Enhanced telomerase activity

The structures at the ends of eukaryotic chromosomes are termed "Telomeres". As telomeres are lost during cell divisions, the chromosomal ends are no longer protected, which leads to the fusion of the chromosomes and karyotypic abnormalities, that eventually cause cell death. The ribonucleoprotein enzyme telomerase extends the telomeric repeat sequences at the chromosomal ends; and it is active in a majority (90%) of human neoplasia, but inactive in most normal cells.[36] Mao *et al* analyzed 16 HNSCC cell lines, 29 tumor specimens and adjacent normal and dysplastic mucosa; and did not find telomerase activity in any of the normal tissues, but it was found in 100% of the cell lines, 90% of the invasive neoplasia and 100% of the dysplastic lesions.[37] Other reports also confirm high telomerase activity in oral tumors[38] and in 37% of OSCC patients, using oral rinses.[39]

Neovascularization

Angiogenesis, defined as the growth of new blood vessels (neovascularization) from pre-existing ones, is a complex process, absolutely needed for the continued growth and survival of solid neoplasia. This process of angiogenesis is in itself a multi-step process, that appears to be regulated by both stimulatory and inhibitory factors.[40] The steps critical to successful angiogenesis, include the degradation of the extracellular matrix, endothelial cell proliferation, migration and assembly of endothelial cells into higher order structures. In the majority of cancers, highly vascularized tumors showed a poor prognosis and the influence of tumor angiogenesis proved to be independent of conventional prognostic indicators.[41]

Cytokine profile

Suppression of *Th1* cytokine genes, was reported due to increase in tumor load and lymph node invasion, which skewed it towards a *Th2*-like cytokine response. Mehrotra *et al* have earlier reported that HNSCCs, but not benign lesions, express Interleukin-4 receptors *in situ*[42] and Interleukin-13 was secreted by HNSCCs, but does not modulate their growth *in vitro*. It has been postulated that these cytokines, in addition to others, were responsible for the growth pattern of these tumors and could be responsible for their active spread.[43]

Proteomics and array technology

Oncologic research during the past decade, relied on the use of genomic tools and now, in the post genomic era, there is a strong drive towards proteomics. Genomics and proteomics studies are used for the molecular classification of tumors and the identification of markers for the early detection of cancer. The National Cancer Institute, Bethesda, USA has successfully completed the Cancer genome anatomy project (CGAP), with the goal of achieving a comprehensive molecular characterization of normal, pre-cancerous and malignant cells, to create a complete profile of genes expressed during cancer development.[44]

The term "Proteomics" indicates proteins expressed by a genome and is the systematic analysis of protein profiles of tissues. In a recent report, an oral cancer-specific human Bacterial artificial chromosome (BAC) array, called the oral cancer genomic regional array (OCGR), has also been described to detect copy number alterations in OSCC.[45] BAC array CGH is similar to conventional chromosomal CGH, except that it uses segments of human DNA as hybridization targets,

instead of a metaphase spread of chromosomes. Hybridization on to such arrays, overcomes the low resolution that limits conventional CGH. As with conventional CGH, total genomic DNA from a tumor and a normal cell population are differentially labeled and co-hybridized onto an array. The ratio of the fluorescence intensities on each DNA spot on the array is proportional to the copy number of the corresponding sequence. High-resolution arrays allow for the delineation of amplification and deletion boundaries, in a single experiment.

These arrays have been instrumental in detailed analysis of specific chromosomal regions. Mendez *et al* examined the genetic expression profiles of 26 invasive squamous cell carcinomas of the oral cavity and oropharynx, 2 premalignant lesions and 18 normal oral tissue samples, using oligonucleotide arrays representing approximately 7000 human genes and found many genes to be up and down regulated, thus helping to differentiate the premalignant from the malignant tissues.[45]

Leethanakul *et al* compared the gene expression profiles of five laser- microdissected head and neck squamous cell carcinioma (HNSCC) specimens, with those of their matching normal tissues.[46] In the study, human cancer cDNA membrane arrays from *Clontech* USA were used, containing DNA fragments in duplicate, for 588 known human cancer genes. Interestingly, the genes expressed differentially in the invasive specimens compared with their corresponding normal tissues and included those involved in the control of cell growth and differentiation, angiogenesis, apoptosis, cell cycle and signaling, most of which have not been previously described in HNSCC. Belbin *et al*, have identified 375 genes that demonstrated significant expression differences and divided the patients with HNSCCs into clinically distinct subgroups, based on gene expression patterns found and outcome could be predicted.[47]

The development of high throughput hybridisation based methods, such as cDNA and oligonucleotide microarrays, allows the simultaneous analysis of gene expression alterations in thousands of genes, in HNSCC. These studies have used a variety of experimental designs, tumour types, array platforms and statistical tools, making direct comparisons of results difficult. However, the potential biological and clinical implications of gene expression signatures of HNSCC in tumour classification, prognosis and treatment, are enormous.[48]

Another approach using Polymerase chain reaction differential display technique, has been applied by Lemaire *et al*.[49] Contrary to the microarrays, wherein analysis is limited to the set of genes printed on the array, the Polymerase chain reaction differential display technique has the advantage of randomly sampling the whole transcriptome. Using this technique, the authors were able to identify novel expressed sequences, that did not correspond to known genes, which could be potential tumor markers and targets for drug design in HNSCC.

New evidence for new gene expression- based biomarkers of local treatment failure in the head and neck cancers, was reported recently by Ginos *et al,* who studied gene expression profile in 41 cases and found 2890 genes associated with extracellular matrix production, proliferation, cytokine/chemokine expression, immune response, invasion and metastasis that showed significant differences in their expression.[50] Another new protein analysis system based on the surface enhanced laser desorption/Ionization (SELDI), has been recently applied for the separation, detection and analysis of multiple proteins in a very small amount (~10ng) of micro-dissected cancer tissue. This technique facilitates protein capture, purification, analysis and processing from complex biological mixtures, directly onto protein chip array surfaces and the detection of the purified proteins is performed by Time of Flight Mass Spectrometry, (TOF-MS). A major feature in SELDI-TOF MS, is its ability to provide a rapid protein expression profile.[51]

Laser Capture Microdissection (LCM)

This novel technology offers sample purity and complements gene chips, by allowing accurate gene profiling studies. The method of LCM has made the study of cancer biology and other areas of cell biology, more precise and has greatly boosted the current efforts in defining the molecular basis of malignancy, as they exist *in vivo*.[42] The microenvironment of a carcinoma consists not only of the malignant epithelial component, but also the surrounding stroma and normal tissue. These distinct microcompartments use receptors, cell junctions and inter and intracellular signaling molecules, to allow tumor cells to communicate with their surroundings and play an active role in their own control or progression. LCM provides an ideal method for the extraction of cells from specimens, in which the exact morphologies of both the captured cells and the surrounding tissue, are preserved. When rapid immunohistochemical staining techniques are combined with LCM, we can get more accurate microdissection of cell subsets.[52]

New Therapies

Management of cancer remains difficult, in spite of considerable advances in understanding the molecular biology of oral cancer and still we bank upon radiotherapy and chemotherapy. Although more than 1500 anticancer agents are in active development, with over 500 in clinical trials, still there is a desperate need for more effective and less toxic therapies. Therapeutic agents targeted specifically at patients with head and neck cancer, include the family of tyrosine-kinase inhibitors in particular, the epidermal growth factor-receptor (EGFR) and cyclin dependent kinase (CDK) inhibitors. Promising gene therapy strategies have been reported on the use of highly efficient adenovirus vectors, to deliver therapeutic genes in advanced cases of HNSCC. A prime example is ONYX-015, which is an adenovirus with the EIB55-kDa gene deleted, to selectively replicate in and lyse p53-deficient cancer cells, while sparing normal cells. A combination of intratumoural ONYX-015 injection with Cisplatin and 5-Flourouracil in patients, suggests that it is effective and that the response at injected tumor sites is durable.[53] "Irressa" is an orally-active selective epidermal growth factor receptor-tyrosine kinase inhibitor, showing anti-tumor activity in HNSCC, in combination with radiation treatment.[54]

Onco-chips are the new concept consisting of several reliable diagnostic head and neck cancer markers, which may be used to diagnose cancer. The treatment of cells with therapeutic chemicals, has been shown to produce specific changes in gene expression and owing to the costly nature of clinical trials involved, much effort by pharmaceutical companies is being invested into "*Toxo chips",* which may contain the relevant probes to study cell expression responses to chemical or drug insult, during drug development.[54]

Conclusion

The global increase in frequency and mortality, as well as the poor prognosis of head and neck squamous cell carcinoma, has intensified current research efforts in the field of prevention and early detection of this disease. The advances in the understanding of the molecular basis of HNSCC should help in the identification of new markers. The study of the carcinogenic process of the head and neck, including continued analysis of new genetic alterations, along with their temporal sequencing during initiation, promotion and progression, will allow us to identify new diagnostic and prognostic factors, which will provide a promising basis for the application of more rational and efficient treatments.

References

1. Landis SH, Murray T, Bolden S, Wingo PA, Cancer statistics, 1999. CA Cancer J Clin 1999;49:8-31.
2. Mehrotra R, Gupta A, Singh M, Ibrahim R. Application of cytology and molecular biology in diagnosing premalignant or malignant oral lesions. Mol Cancer 2006;5:11.
3. Meyskens FL. Biology and intervention of premalignant process. Can Bull 1991;43:475-80.
4. Sunny L, Yeole BB, Hakama M, Shiri R, Sastry PS, Mathews S, *et al*. Oral cancers in Mumbai, India: A fifteen years perspective with respect to incidence trend and cumulative risk. Asian Pac J Cancer Prev 2004;5:294-300.
5. Daftary DK, Murti PR, Bhonsle RB, *et al*. Risk factors and risk markers for oral cancer in high incidence areas of the world. *In*: Johnson NW, editor. Oral Cancer, Vol. 2. Cambridge University Press: Cambridge; 1991. p. 29-63.
6. Walker DM, Boey G, McDonald LA. The pathology of oral cancer. Pathology 2003;35:376-83.
7. Franceschi S, Bidoli P, Herrero R, Munoz N. Comparison of cancers of the oral cavity and pharynx world wide:etiological clues. Oral Oncol 2000;36:106-15.
8. Manjari M, Popli R, Paul S, Gupta VP, Kaholon SK. Prevalence of oral cavity, pharynx, larynx and nasal cavity malignancies in Amritsar, Punjab. Indian J Otolaryngol Head Neck Surg 1999;48:191-5.
9. International Agency for Research on Cancer (IARC). Tobacco smoking. IARC Monograph on the evaluation of carcinogenic risks of chemicals to humans. IARC: Lyon, France; 1986. p. 1-421.
10. Kawajiri K, Fujii-Kuriyama Y. P450 and human cancer. Jpn J Cancer Res 1991;82:1325-35.
11. Merchant A, Husain SS, Hosain M, Fikree FF, Pitiphat W, Siddiqui AR, *et al*. Paan without tobacco: An independent risk factor for oral cancer. Intl J Cancer 2000;86:128-31.
12. Nair J, Ohshima H, Friesen M, Croisy A, Bhide SV, Bartsch H. Tobacco-specific and betel nut-specific N-nitroso compounds: Occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid. Carcinogenesis 1985;6:295-303.
13. Prokopczyk B, Rivenson A, Bertinato P, Brunnemann KD, Hoffmann D. 3-(Methylnitrosamino) propionitrile: Occurrence in saliva of betel quid chewers, carcinogenicity and DNA methylation in F344 rats. Cancer Res 1987;47:467-71.
14. International Agency for Research on Cancer (IARC). Alcohol drinking. IARC Monograph on the evaluation of carcinogenic risks of chemicals to humans. IARC: Lyon, France; 1989. p. 1-416.
15. Blot WJ. Alcohol and cancer. Cancer Res 1992;52:2119s-23s.
16. Harty LC, Caporaso NE, Hayes RB, Winn DM, Bravo-Otero E, Blot WJ, *et al*. Alcohol dehydrogenase 3 genotype and risk of oral cavity and pharyngeal cancers. J Natl Cancer Inst 1997;89:1698-705.
17. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, *et al*. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92:709-20.
18. McKaig RG, Baric RS, Olshan AF. Human papillomavirus and head and neck cancer: Epidemiology and molecular biology. Head Neck 1998;20:250-65.
19. DiPaolo JA, Woodworth CD, Popescu NC, Koval DL, Lopez JV, Doniger J. HSV-2 induced tumorigenicity in HPV-16-immortalised human genital keratinocytes. Virology 1990;177:777-9.
20. Scully C. New aspects of oral viral diseases. Curr Top Pathol 1996;90:29-96.
21. Scully C. Oral squamous cell carcinoma; From a hypothesis about a virus, to concern about possible sexual transmission. Oral Oncol 2002;38:227-34.
22. McLaughlin JK, Gridley G, Block G, Winn DM, Preston-Martin S, Schoenberg JB, *et al*. Dietary factors in oral and pharyngeal cancer. J Natl Cancer Inst 1988;80:1237-43.

23. De Stefani E, Deneo-Pellegrini H, Mendilaharsu M, Ronco A. Diet and risk of cancer of the upper aerodigestive tract-I. Foods. Oral Oncol 1999;35:17-21.
24. Negri E, Franceschi S, Bosetti C, Levi F, Conti E, Parpinel M, *et al*. Selected micronutrients and oral and pharyngeal cancer. Int J Cancer 2000;86:122-7.
25. Spitz MR, Fueger JJ, Beddingfield NA, Annegers JF, Hsu TC, Newell GR, *et al*. Chromosome sensitivity to bleomycin induced mutagenesis an independent risk factor for upper aerodigestive tract cancer. Cancer Res 1989;49:4626-8.
26. Villaret DB, Wang T, Dillon D, Xu J, Sivam D, Cheever MA, *et al*. Identification of genes over expressed in head and neck squamous cell carcinoma using a combination of complementary DNA subtraction and microarray analysis. Laryngoscope 2000;110:374-81.
27. Scully C, Laskaris G, Pindborg J, Porter SR, Reichart P. Oral manifestations of HIV and their management. I. More common lesions. Oral Surg Oral Med Oral Pathol 1991;71:158-66.
28. Flaitz CM, Hicks MJ. Role of lymphotrotrophic herpesvirus in malignancies associated with immunosuppressed states. *In*: Millard HD, Mason DK, editors. Perspectives on the 1998 3rd World Workshop on Oral Medicine. University of Michigan Press: Ann Arbor, MI; 2000.
29. Harris JP, Penn I. Immunosuppression and the development of malignancies of the upper airway and related structures. Laryngoscope 1981;91:520-8.
30. Cawson RA. Leukoplakia and oral cancer. Proc R Soc Med 1969;62:610-4.
31. Mehrotra R, Vasstrand EN, Ibrahim SO. Recent advances in understanding carcinogenicity of oral squamous cell carcinoma: From basic molecular biology to latest genomic and proteomic findings. Cancer Gen Proteom 2004;1:283-94.
32. Weinberg RA. Tumor suppressor genes. Science 1991;254:1138-46,52.
33. Schepman KP, van der Waal I. A proposal for classification and staging system for oral leukoplakia: A preliminary study. Oral Oncol 1995;31B:396-8.
34. Grandis JR, Tweardy D. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 1993;53:3579-84.
35. Teni TR, Pawar SN, Mallick S. Expression of Apoptosis related genes in human oral cancers from India. 3rd World assembly on tobacco counters health, Book of Abstracts: 2004. p. 36.
36. Reddel RR. Alternative lengthening of telomeres, telomerase and cancer. Cancer Lett 2003;194:155-62.
37. Mao L, Lee JS, Fan YH, Ro JY, Batsakis JG, Lippman S, *et al*. Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. Nat Med 1996;6:682-5.
38. Miyoshi Y, Tsukinoki K, Imaizumi T, Yamada Y, Ishizaki T, Watanabe Y, *et al*. Telomerase activity in oral cancer. Oral Oncol 1999;35:283-9.
39. Califano J, Ahrendt SA, Meininger G, Westra WH, Koch WM, Sidransky D. Detection of telomerase activity in oral rinses from head and neck squamous cell carcinoma patients. Cancer Res 1996;56:5720-2.
40. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401-10.
41. Kerbel RS. Tumor angiogenesis: Past, present and the near future. Carcinogenesis 2000;21:505-15.
42. Mehrotra R, Varricchio F, Husain SR, Puri RK. Head and neck cancers, but not benign lesions, express interleukin-4 receptors in situ. Oncol Rep 1998;5:45-8.
43. Mehrotra R. Interleukin 13 is secreted by human head and neck tumours and does not modulate their growth in vitro. Indian J Exp Biol 1998;36;805-7.
44. Shillitoe EJ, May M, Patel V, Lethanakul C, Ensley JF, Strausberg RL, *et al*. Genome-wide analysis of oral cancer-Early results from the Cancer Genome Anatomy Project. Genome Anatomy Project. Oral Oncol 2000;36:8-16.
45. Garnis C, Campbell J, Zhang L. Rosin MP, Lam WL. OCGR array: An oral cancer genomic regional array for comparative genomic hybridization analysis. Oral Oncol 2004;40:511-9.
46. Leethanakul C, Patel V, Gillespie J, Pallente M, Ensley JF, Koontongkaew S, *et al*. Distinct pattern of expression of differentiation and growth-related genes in squamous cell carcinomas of the head and neck revealed by the use of laser capture micro dissection and cDNA arrays. Oncogene 2000;19:3220-4.
47. Belbin TJ, Singh B, Barber I, Socci N, Wenig B, Smith R, *et al*. Molecular classification of head and neck squamous cell carcinoma using cDNA microarrays. Cancer Res 2002;62:1184-90.
48. Mendez E, Cheng C, Farwell DG, Ricks S, Agoff SN, Futran ND, *et al*. Transcriptional expression profiles of oral squamous cell carcinomas. Cancer 2002;95:1482-94.
49. Lemaire F, Millon R, Young J, Cromer A, Wasylyk C, Schultz I, *et al*. Differential expression profiling of head and neck squamous cell carcinoma (HNSCC). Br J Cancer 2003;86:1940-9.
50. Ginos MA, Page GP, Michalowicz BS, Patel KJ, Volker SE, Pambuccian SE, *et al*. Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. Cancer Res 2004;64:55-63.
51. Marvin LF, Roberts MA, Fay LB. Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry in clinical chemistry. Clin Chim Acta 2003;337:11-21.
52. Fend F, Emmert-Buck MR, Chuaqui R, Cole K, Lee J, Liotta LA *et al*. Immuno-LCM: Laser capture micro dissection of immunostained frozen sections for mRNA analysis. Am J Pathol 1999;154:61-6.
53. Khuri FR, Nemunaitis J, Ganly I, Arseneau J, Tannock IF, Romel L, *et al*. A controlled trial of intratumoral ONYX-015, a selectively replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. Nature Med 2000;6:879-85.
54. Dykxhoorn DM, Novina CD Sharp PA. Killing the messenger: Short RNAs that silence gene expression. Nat Rev Mol Cell Biol 2003;4:457-67.