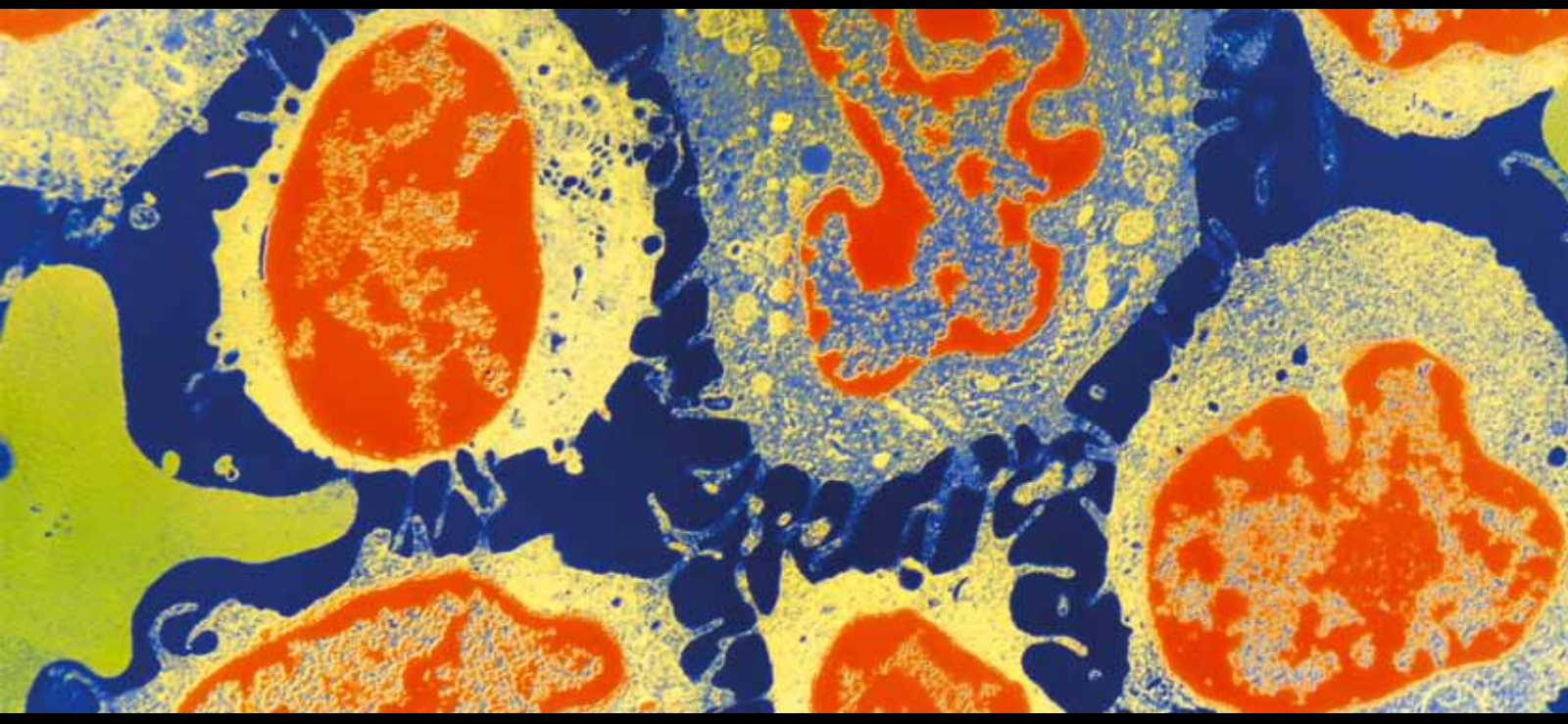
Journal of Oncology

Prevention and Early Detection of Head and Neck Squamous Cell Cancers

Guest Editors: Toru Nagao, Pankaj Chaturvedi, Ashok Shaha, and Rengaswamy Sankaranarayanan



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Editorial Board

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Editorial

Prevention and Early Detection of Head and Neck Squamous Cell Cancers

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Head and neck squamous cell cancer (HNSCC) is the sixth most common malignancy reported worldwide and one with high mortality ratios among all malignancies. Unfortunately 5-year survival rate has not improved (50% overall) for the last few decades except in specialized cancer centres. Oral and HNSCC patients come at late stage due to their own delay as well as professional delay at primary and specialized care levels. Efficacious preventive strategies, educations, and early detections may have the capacity for HNSCC and potentially malignant disorders to be detected at an asymptomatic phase. For primary and secondary cancer prevention, changing lifestyle is an integral part of health promotion interventions, particularly among high risk group. Chemoprevention is an ideal one to lower the chance of getting cancer. Thus the early detection and subsequent intervention may achieve a significant reduction of mortality rate in this population.

This special issue about prevention and early detection of HNSCC is all in our minds, and it stresses on current topics of epidemiology, diagnosis, tumour markers, and chemoprevention for oral and HNSCC. All eight papers are review articles from various parts of the world where oral and HNSCCs are major public health issues, although the incidence is not high.

With regards to tumour markers, no significant serological markers are available so far that would be helpful in detecting primary HNSCCs at early stage, but the most widely accepted biomarker for HNSCCs is high-risk HPV status. The incidence of oropharyngeal SCC is rising, and the Agency for Research on Cancer (IARC) now recognizes HPV as a risk factor for oropharyngeal cancer. L. Marklund and L. Hammarstedt present a current advancement of HPV studies in HNSCC: HPV biology, oncogenic mechanisms, risk factors, epidemiology, and clinical implications. HPV-positive oropharyngeal cancer is recognized as a distinct subset of HNSCC with a favourable outcome, and patients with HPV-positive oropharyngeal cancers often are younger and in good health. The authors suggest that further knowledge about tumour biology and the identification of additional clinical useful markers is needed to combine with HPV status for appropriate risk stratification in future clinical trials in order to optimize the treatment for each individual patient. X. Li et al. present an inhibitor of growth gene (*ING*) family consisting of five genes, from *ING1* to *ING5*, identified as a new tumour suppressor gene family. These *ING* family genes are supposed to belong to type II tumour suppressor gene and are involved in multiple cellular processes including chromatin remodeling, DNA repair, cell cycle control, senescence, and apoptosis. The authors conclude that the *ING* gene family could be a novel p53-independent biomarker for HNSCC.

T. Tanaka and R. Ishigamori provide a review of the detection of high risk patients by potential biomarkers for oral carcinogenesis such as epidermal growth factor receptor (EGFR), which plays critical roles in HNSCC carcinogenesis and others well-known ones as well as chemoprevention. Individualized medical therapy to specific genetic abnormalities detected within the oral mucosa is a promising approach. M. Masuda et al. present a potential of green tea extract, (-)-epigallocatechin-3-galate (EGCG), in HNSCC

chemoprevention and the role of EGFR signaling and lipid raft. The authors show the inhibition of EGFR by EGCG and the important role of lipid raft that emerged as an important platform of numerous biophysical functions such as receptor tyrosine kinase signaling including EGFR. Y. Zhao et al. present a Salvianolic acid B (Sal-B), which is a well-known Chinese medicine used to treat and prevent aging diseases for thousands of years and significantly inhibits or delays the growth of HNSCC in both cultured HNSCC cells and HNSCC xenograft animal models. Anticancer mechanisms such as inhibition of COX-2/PGE-2 pathway, promotion of apoptosis, and modulation of angiogenesis are proposed, and it is concluded that Sal-B is a potential HNSCC chemopreventive agent working through antioxidation and anti-inflammation mechanisms.

The adjunct diagnostic technique is important for early detection mostly at primary care level prior to tissue biopsy. S. F. Mendes et al. present a review of diagnostic techniques for oral potentially malignant disorders and oral exfoliative cytology, cytomorphometry, tissue staining, chemiluminescence, and light emission technique. A. Böcking et al. provide a useful method of brush biopsy and DNA image cytometry as screening tools for prevention, diagnosis, therapy, and follow-up care of oral cancer and precursor lesions. The authors suggest that the diagnostic DNA image cytometry is an accurate method and has internationally been standardized and noted that it is paid by the German health insurances. C. Szeto et al. try to review the efficacy of contact endoscopy by literature searches in early diagnosis, monitoring, and preoperative assessment of mucosal lesions of HNSCC.

Oral and HNSCC is one of the lethal diseases in all cancers, and their natural history is still further behind. We hope that this issue may inspire the development of new strategies and policies for early detection and subsequent intervention for HNSCC in order to get better outcome of cancer prevention and treatment and, consequently, reduce the mortality rate.

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Review Article

Understanding Carcinogenesis for Fighting Oral Cancer

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Oral cancer is one of the major global threats to public health. Oral cancer development is a tobacco-related multistep and multifocal process involving field cancerization and carcinogenesis. The rationale for molecular-targeted prevention of oral cancer is promising. Biomarkers of genomic instability, including aneuploidy and allelic imbalance, are able to measure the cancer risk of oral premalignancies. Understanding of the biology of oral carcinogenesis will give us important advances for detecting high-risk patients, monitoring preventive interventions, assessing cancer risk, and pharmacogenomics. In addition, novel chemopreventive agents based on molecular mechanisms and targets against oral cancers will be derived from research using appropriate animal carcinogenesis models. New approaches, such as interventions with molecular-targeted agents and agent combinations in high-risk oral individuals, are undoubtedly needed to reduce the devastating worldwide consequences of oral malignancy.

1. Introduction

Head and neck cancer is the sixth most common human cancer [1], representing 3% of all types of cancer. They are located in the oral cavity in 48% of cases, and 90% of these are oral squamous cell carcinoma [2]. They are sometimes preceded by precancerous lesions, such as leukoplakia and erythroplakia. More than 300,000 new cases worldwide are being diagnosed with oral squamous cell carcinoma annually [3]. Approximately 35,000 new cases are recorded annually in the US [2], 40,000 new cases in the EU, and 10915 new cases in Japan [4]. The most common site for intraoral carcinoma is the tongue, which accounts for around 40% of all cases in the oral cavity proper. Tongue cancers most frequently occur on the posteriorlateral border and ventral surfaces of the tongue. The floor of the mouth is the second most common intraoral location. Less common sites include the gingival, buccal mucosa, labial mucosa, and hard plate.

The incidence of oral cancer has significant local variation. In India and other Asian countries, oral and pharyngeal carcinomas comprise up to half of all malignancies, with this particularly high prevalence being attributed to the influence of carcinogens and region-specific epidemiological factors, especially tobacco and betel quid chewing. An increase in oral cancer prevalence among young adults is a cause of special concern. There has been a 60% increase in the number of under 40 years old with tongue cancer over past 30 years. However, few data have been published on the etiology and natural history of this increase [5]. Oral malignancy including tongue cancer is associated with severe morbidity and less than 50% long-term survival despite advances in treatment (surgery, radiation, and chemotherapy) of oral cancer. The survival of the patients remains very low, mainly due to their high risk of developing a second primary cancer. Thus, early detection and prevention of oral cancer and premalignancy are quite important [6–10]. This paper will focus on our understanding of oral carcinogenesis for preventing and early detection of oral malignancy.

2. Oral Carcinogenesis

Oral carcinogenesis is a highly complex multifocal process that takes place when squamous epithelium is affected by

several genetic alterations. The use of several molecular biology techniques to diagnose oral precancerous lesions and cancer may markedly improve the early detection of alterations that are invisible under the microscope. This would identify patients at a high risk of developing oral cancer [11]. Natural history of oral cancer and sequence of genetic alterations are illustrated in Figure 1. There are approaches to understanding of the molecular basis of oral cancer [12–14]. They include microarray technology, methylation microarrays, gene expression microarrays, array comparative genomic hybridization, proteomics, mitochondrial arrays, and micro-RNA arrays [15]. To date, high-throughout approaches are being used to search for oral cancer biomarkers in biofluids (saliva and serum) [15].

"Field cancerization" refers to the potential development of cancer at multiple sites [16, 17]. This has been observed during the development of cancer in the tissues covered with squamous epithelium (head and neck tumor) and transitional epithelium (urothelial carcinoma). It is evident that oral cancer, like carcinomas in other tissues, develops over many years, and during this period, there are multiple sites of neoplastic transformation occurring throughout the oral cavity. Mutations of this gene have been observed in various sites of premalignant leukoplakia and carcinoma in the same oral cavity [18]. A reduction in tumor suppressor activity by the gene and the development of mutations in *p53* have been associated with smoking and an increased risk for oral carcinoma development [19]. Therefore, multifocal presentations and mutational expressions of tumor suppressor genes may be the consequence of long-term (e.g., 20∼40 years) exposure to various environmental and exogenous factors. The continual presence of mutations may also signify changes in DNA repair and apoptosis, thereby increasing the susceptibility for future transformation. Mutational adaptations that modify the survivability of particular clones of transforming cells may also further enhance the level of resistance to therapeutic control. Recent genetic analysis has revealed that cancers developing at distant sites within the oral cavity often are derived from the same initial clone [20]. The multiplicity of the oral carcinogenesis process makes it difficult to interrupt the progression to cancer through surgical removal of a premalignant lesion.

3. Risk Factors of Oral Cancer

The most important risk factor for the development of oral cancer in the Western countries is the consumption of tobacco [21] and alcohol [22]. Although drinking and smoking are independent risk factors, they have a synergistic effect and greatly increase risk together. In Asian countries, the use of smokeless tobacco products such as gutkha and betel quid [5, 23] is responsible for a considerable percentage of oral cancer cases. Several studies have reported a significant familial component in the development of oral cancer. The estimates of risk in the first degree relatives of oral cancer patients vary widely and have been reported to be 1.1 [24] ∼ 3.8 [25] although some of these refer to head and neck cancer in general. Familial aggregation of oral cancer, possibly with an autosomal dominant mode of inheritance, was reported in a very small percentage of oral cancer patients [26]. Polymorphic variation of genes in the xenobiotic metabolism pathways, such as in *CYP1A1* or the genes coding for glutathione *S*-transferase-M1 [27, 28] and *N*-acetyltransferase-2 [29] may be implicated. Individuals that carry the fast-metabolizing alcohol dehydrogenase type 3 (*ADH3*) allele [30] may be particularly vulnerable to the effects of chronic alcohol consumption and could be at increased risk to develop oral cancer [31].

Human papilloma virus (HPV), particularly HPV type 16, may be an etiologic factor, especially among persons who do not smoke or drink alcohol [32, 33]. Ang et al. [34] reported that tumor HPV status is a strong and independent prognostic factor for survival among patients with oropharyngeal cancer. They also noted that the risk of death significantly increased with each additional pack year of tobacco smoking. Although the idea that bacterial infections could lead to oral cancer has not been well regarded, there recently has been an increasing body of evidence to suggest a possible relationship between microorganisms and oral cancer development. The most notable example is that of the common pathogenic bacterium *Helicobacter pylori* and its association with gastric cancer. The mouth comprises a variety of different surfaces that are home to a huge diversity of microorganisms, including more than 750 distinct taxa of bacteria, suggesting that the oral squamous epithelium is constantly exposed to a variety of microbial challenges, on both cellular and molecular levels. In this context, we should draw attention to how they may relate to oral cancer development [35, 36].

There are clinically apparent oral premalignant lesions of oral cancer. They include leukoplakia, erythroplakia, nicotine stomatitis and tobacco pouch keratosis, lichen planus, and submucous fibrosis [37]. The term "leukoplakia" first used by Schwimmer in 1877 [38] to describe a white lesion of the tongue probably represented a syphilitic glossitis. The definition of leukoplakia has often been confusing and controversial. Some clinicians now avoid using this term. As defined by the World Health Organization, leukoplakia is "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease [39]". As such, leukoplakia should be used only as a clinical term. The term has no specific histopathological connotation and should never be used as a microscopic diagnosis. In the evaluation of the patient, leukoplakia is a clinical diagnosis of exclusion. Sometimes, a white patch is initially believed to represent leukoplakia, but the biopsy reveals another specific diagnosis. In such cases, the lesion should no longer be categorized as a leukoplakia. Leukoplakia is seen most frequently in middle-aged and older men, with an increasing prevalence with age [40]. Fewer than 1% of men below the age of 30 have leukoplakia, but the prevalence increases to an alarming 8% in men over the age of 70 [40]. The prevalence in women past the age of 70 is approximately 2%. The most common sites are the buccal mucosa, alveolar mucosa, and lower lip. However, lesions in the floor of mouth, lateral tongue, and lower lip are most likely to show dysplastic or malignant changes [41].

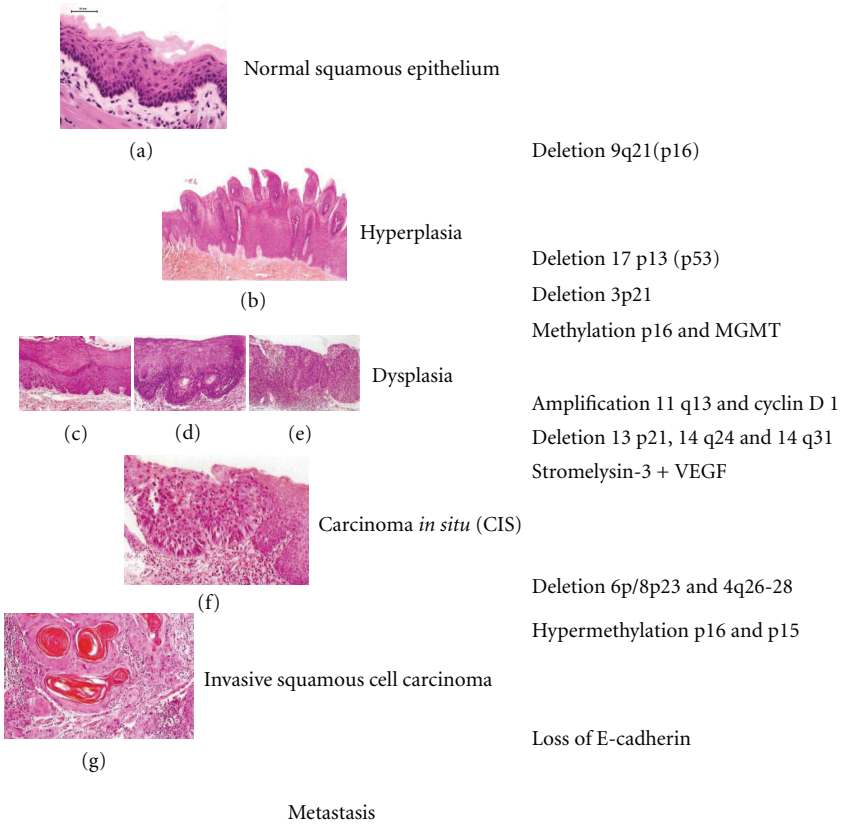

Figure 1: Natural history and genetic alterations of oral carcinogenesis. (a), Normal oral mucosa, (b) papillary hyperplasia, (c) midl dysplasia, (d) moderate dysplasia, (e) severe dysplasia, (f) carcinoma in situ, and (g) invasive squamous cell carcinoma (well differentiated).

The term "erythroplasia" originally used by Queyrat [42] to describe a red, precancerous lesion of the penis is used for a clinically and histopathologically similar process that occurs on the oral mucosa. Similar to the definition for leukoplakia, erythroplakia is a clinical term that refers to a red patch that cannot be defined clinically or pathologically as any other condition [39]. This definition excludes inflammatory conditions that may result in a red clinical appearance. Oral erythroplakia occurs most frequently in older men and appears as a red macule or plaque with a soft, velvety texture. The floor of mouth, lateral tongue, retromolar pad, and soft palate are the most common sites of involvement. Often the lesion is well demarcated, but some examples may gradually blend into the surrounding mucosa. Some lesions may be intermixed with white areas (erythroleukoplakia). Erythroplakia is often asymptomatic although some patients may complain of a sore, burning sensation.

Nicotine stomatitis is a thickened, hyperkeratotic alteration of the palatal mucosa that is most frequently related to pipe smoking, but milder examples can also develop secondary to cigar smoking or, rarely, from cigarette smoking [39]. The palatal mucosa becomes thickened and hyperkeratotic, sometimes developing a fissured surface. The surface often develops popular elevations with red centers, which represent the inflamed openings of the minor salivary gland ducts.

Detection and diagnosis of oral neoplasia has traditionally relied heavily on the clinical experience of the examiners and their ability to recognize often subtle morphologic changes. However, some early malignant lesions are clinically indistinguishable from benign lesions, and some patients develop carcinomas in the absence of clinically identifiable oral premalignant lesions. Furthermore, it can be difficult even for experts to determine which oral premalignant lesions are at significant risk to progress to invasive carcinoma. Therefore, an accurate, objective, and noninvasive method to help identify premalignant lesions and to distinguish those at risk of malignant conversion is needed.

4. Biomarkers of Oral Cancer

Biomarkers help in the evaluation of prevention or use of therapies and the detection of the earliest stages of oral mucosal malignant transformation. Biomarkers reveal the genetic and molecular changes related to early, intermediate, and late end points in the process of oral carcinogenesis [43]. These biomarkers will refine our ability to enhance the prognosis, diagnosis, and treatment of oral carcinomas [44]. Genetic and molecular biomarkers will also determine the effectiveness and safety of chemopreventive agents. Chemopreventive agents are chemicals of natural or synthetic

origin. Unlike other drugs, which do not prevent disease, chemopreventive agents reduce the incidence of diseases such as cancer before clinical symptoms occur. This development is critical for the understanding of early oral mucosal transformation. Biomarkers will also reduce the number of patients and the time for long-term follow up required to define a significant clinical response to a chemopreventive agent [45, 46]. The markers may, therefore, clarify the types, doses, frequencies, and regimens to achieve the maximum level of benefit from chemopreventive agents. Decreasing the cost of the clinical trials is another factor that drives the development of biomarkers.

Biomarkers have been categorized following the recommendation by the Committee on Biological Markers of the National Research Council/National Academy of Sciences [47]. They fall into broad groups that detect exposure, progression, susceptibility to carcinogens, and/or the responses by the target cellular populations [46].

A distinct advantage to oral cancer studies is their anatomical access to the developing premalignant and malignant lesions. One could readily analyze biopsies of the primary lesion as well as apparently normal mucosal sites to determine the levels of DNA adducts and oral cancer risk. DNA adduct studies and cytogenetic analyses may also provide evidence for altered structure and function of susceptibility sites in the DNA following DNA-binding studies of nuclear proteins such as p53. Some researchers have focused on microscopic cytogenetic and somatic mutation changes as early biologic markers. One of the markers used to define chromosomal aberrations is the staining for micronuclei in exfoliated buccal mucosal cells [48]. Micronuclei have also been used to evaluate the reversal of leukoplakia and the effectiveness of retinoids, carotenoids, and vitamin E [49, 50]. Other methods include the determination of aneuploidy, and the assessment of losses and gains of genetic material particularly associated with somatic and sex chromosomes. Other sites of chromosomal aberrations are found in sister chromatid exchanges, and allele typic variations designated by losses on chromosomes 3, 4, 5, 6, 8, 9, 11, 13, 17, and 19.

Some molecular biomarkers with potential diagnostic relevance include DNA content and chromosome polysomy, loss of heterozygosity, nucleolar organizer regions, histo-blood group antigens, proliferation markers, increased epidermal growth factor receptor (EGFR), and decreased expression of retinoic acid receptor-β, p16, and p53 [51, 52]. Although a reliable, validated marker panel for providing clinically useful prognostic information in oral premalignant lesions patients has not yet been established, the advent of high throughput genomic and proteomic analysis techniques may soon yield major advances toward a prognostically relevant molecular classification system (Table 1).

Table 1: Potential biomarkers for oral carcinogenesis.

Category	Measures
Genomic biomarker	Micronuclei, DNA adduct, DNA content, and chromosomal aberration (polymorphism, alleic loss, gain, and amplification)
Oncogenic biomarker	Oncogenic expression, modified tumor suppressor genes, and *Src* genes
Proliferation biomarker	Nuclear and cyclin-related antigens, mitotic frequency, ornithine decarboxylase (ODC), and polyamines
Differentiation biomarker	Cytokeratins, transglutaminase Type I, and transcription factor (AP)-1
Oxidative stress biomarker	Glutathione *S*-transferase, stress proteins (HSPs), and Superoxide dismutase
Apoptosis biomarker	Bcl-2 family, chromatin condensation factors, caspases, and nucleosome formation
Immunologic biomarker	Cytokines

5. Animal Models for Oral Carcinogenesis

A variety of animals has been used for the study of tumor growth, the process of carcinogenesis and the prevention/treatment research [8, 53–56]. The continual development of transgenic or knockout mice has improved our understanding of the role of specific genes in tumor growth. The most widely used animal models for oral carcinogenesis are the hamster cheek pouch model [54, 57] and the 4-nitroquinoline 1-oxide- (4-NQO-) induced oral (tongue) carcinogenesis model [8, 53, 58, 59].

In the former model, a complete carcinogen, 7,12-dimethylbenz(*a*)anthracene (DMBA, 0.5%), is applied to the hamster cheek pouch three times a week for 16 weeks. By week 16, all animals exhibit invasive oral squamous cell carcinoma. Many different studies have been conducted with the hamster buccal pouch model, and they have provided an array of changes that are analogous to those observed in human invasive oral carcinoma [54, 57]. These include a mutation in codon 61 of Ha-*ras*, which manifested in an A → T transversion in the second position of codon 61, resulting in an amino acid change from glycine to leucine. The expression of c-Ki-*ras* in malignant tumors of the pouch, but not in the normal oral mucosa, has also been observed at very early stages of tumor development [57]. Although the hamster oral tumor model appears to parallel several changes observed in human oral cancer, the hamster still has several areas of uniqueness which must be considered in any evaluations of results from oral carcinogenesis studies. The hamster cheek pouch provides a relatively large surface area of oral mucosa for the development of invasive carcinoma, while the human does not possess this type of mucosal structure. In contrast to humans, mice, or rats, the hamster cheek pouch lacks lymphatic drainage, which allows various drugs or molecules to accumulate in the pouch. The Syrian hamster population was also derived from a small breeding pair that resulted in a restricted polymorphism for the antigen recognition region (Ia region) and some of the major histocompatibility K and D regions [60]. In addition, the number of T-cells in the hamster spleen exhibits a lower number/gram weight of the organ as compared with the mouse or human [60]. The hamster may also respond to

antigenic tumor sources with a natural killer macrophage or granulocyte cytotoxicity rather than a T cell response [60].

The latter animal models for the study of oral carcinogenesis include those in rats and mice using the water-soluble carcinogen, 4-NQO. The carcinogen is either supplied in the water (20 ppm) for the rats [58, 61–74] or by painting for the mice [75]. Administration with 4-NQO in drinking water (20 ppm) for 8 weeks in rats and mice produces tongue lesions including squamous cell neoplasms (Figure 2) within 32 weeks [71], while topical application of the carcinogen to the mouse palates for up to 16 weeks, just like the hamster model develops palate tumors within 49 weeks [75]. Since the most common site for intraoral carcinoma is the tongue and the drinking water administering of 4-NQO is a simple and easy method, the 4-NQO-induced tongue carcinogenesis model is quite useful for investigating oral carcinogenesis and identifying cancer chemopreventive agents [58, 61–74, 76–84]. In the rat model, with the progression of oral carcinogenesis, increased levels of polyamine synthesis have been noted as well as nucleolar organizing regions (NORs) [58].The mouse model with 4-NQO has demonstrated some molecular mimicry of human oral cancers, as is true of the hamster model [75]. A number of chemical carcinogens including coal tar, 20-methylcholanthrene, DMBA, and 4-NQO have been used in experimental oral carcinogenesis. However, 4-NQO is the preferred carcinogen apart from DMBA in the development of experimental oral carcinogenesis. 4-NQO is a water-soluble carcinogen, which induces tumors predominantly in the oral cavity. It produces all the stages of oral carcinogenesis and several lines of evidences suggest that similar histological as well as molecular changes are observed in the human system. There are several review articles to collate the information available on mechanisms of action of 4-NQO, and studies have been carried out for the development of biomarkers and chemopreventive agents using 4-NQO animal models [8–10, 53, 58, 59, 61–68, 70–74].

The complexity and variety of biochemical changes can increase tumor development is the $p53^{-/-}$ mice [85]. Unfortunately, this model and other genetic mouse models have not been exploited for studying the relationships among chemical oral carcinogenesis, specific genetic defects, and chemoprevention. Genetically altered mouse and rat models have been developed for evaluating molecular-targeted prevention and treatment of oral carcinoma [56]. We have developed *ras*H2 transgenic mouse carcinogenesis model [86] and human c-Ha-*ras* proto-oncogene transgenic rat model [87] for chemoprevention studies on oral (tongue) carcinogenesis.

6. Chemoprevention

Chemoprevention is the use of natural or synthetic substances to halt, delay, or reverse malignant progression in tissues at risk to develop invasive cancer [8–10]. Retinoids are the most extensively studied agents for chemoprevention of oral cancer [88]. 13-*cis*-retinoic acid given for only 3 months produced a clinical response rate of 67% versus 10% for placebo. However, toxicities were considerable, and a very high rate of relapse within 3 months of stopping treatment was reported. Subsequent studies with retinoids in patients with oral premalignant lesions have confirmed clinical and pathologic response rates though toxicities remain a concern [89]. However, translational studies showed that molecular abnormalities persisted in some patients with complete clinical and pathologic response to retinoid therapy [90], suggesting that cancer development may be delayed rather than prevented by these agents. Other agents that have been assessed in clinical trials for chemoprevention activity in oral leukoplakia patients include vitamin E [44], Bowman-Birk inhibitor concentrate (BBIC) derived from soybeans [91], curcumin [92], and green tea polyphenol epigallocatechin-3-gallate. Small clinical trials using oral BBIC revealed no significant toxicity and a 32% response rate [91].

Attention is focused now on the development of agents targeted to specific steps in the molecular progression from normal to oral premalignancy to invasive carcinoma. Examples of molecularly targeted agents that have shown promise in vitro, in animal models, or in early clinical trials include cyclooxygenase (COX)-2 inhibitors and epidermal growth factor receptor EGFR inhibitors [93–95]. Data from several sources suggest that the cyclooxygenase pathway is a good target for oral cancer prevention. COX-2 is overexpressed in head and neck squamous carcinoma [96], and COX-2 inhibitors prevented oral cancer development in animal models [97]. A randomized placebo-controlled trial of the COX-2 inhibitor ketorolac administered as an oral rinse in oral leukoplakia patients revealed that the treatment was well tolerated but did not result in greater clinical response than placebo [98]. However, analysis of the results of this trial are confounded somewhat by the high response rate (32%) in the placebo arm and difficulty in determining whether topical delivery of the agent allowed penetration to the damaged cells. The future of COX-2 inhibitors as chemoprevention agents will also depend on the determination of the extent of risk for cardiac toxicities associated with this class of agents. The EGFR is also a promising molecular target for intervention in oral malignant progression [93–95]. EGFR is a receptor tyrosine kinase that is overexpressed in oral dysplasia and invasive cancer and associated with worse prognosis in patients with head and neck squamous carcinoma [99, 100]. EGFR inhibitors, alone or in combination with chemotherapy and radiotherapy, have shown activity against head and neck squamous carcinoma in clinical trials, and toxicities were generally well tolerated [101]. Evidence has suggested that combination therapy targeting COX-2 and EGFR may be efficacious [95, 102]. Although chemoprevention appears to be a promising approach to managing oral premalignancy, prospective clinical trials using specific agents, and strong corollary translational and laboratory investigations, are needed to evaluate clinical, histologic, and molecular efficacy. In the future, it may be possible and necessary to individualize medical therapy to specific genetic abnormalities detected within the oral mucosa.

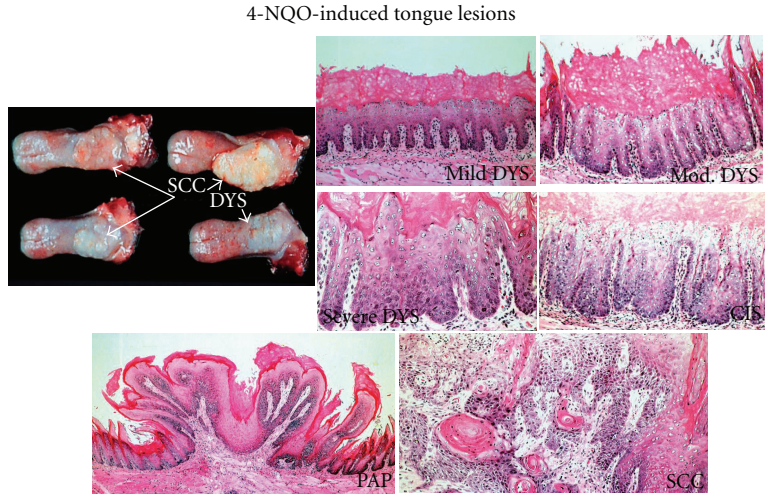

Figure 2: 4-NQO-induced tongue lesions in rats. 4-NQO, 4-nitroquinoline 1-oxide; DYS, dysplasia; PAP, papilloma; SCC, squamous cell carcinoma.

7. Conclusion

Human oral cancer being the sixth largest group of malignancies worldwide. Seventy percent of oral cancers appear from premalignant lesions. The process of oral cancer formation results from multiple sites of premalignant change in the oral cavity (field cancerization). Animal models are being widely used, aiming for the development of diagnostic and prognostic markers. The appearance of these premalignant lesions is one distinct feature of human oral cancer. At present, there is dearth of biomarkers to identify which of these lesions will turn into malignancy. Regional lymph node metastasis and locoregional recurrence are the major factors responsible for the limited survival of patients with oral cancer. Paucity of early diagnostic and prognostic markers is one of the contributory factors for higher mortality rates. Determining high- and low-risk populations by measuring reliable biomarkers help us to understand the dynamics and prevention of oral cancer development. The quantitation of genetic and molecular changes and the use of these changes as markers for the detection and prevention of early premalignant change require the harvesting of tissues and cells. Promising technologies are being rapidly developed to assist in localization of abnormal oral mucosa, in noninvasive and objective diagnosis and characterization of identified mucosal lesions, and in therapy of patients with oral cancer. Undoubtedly, the prevention or reduction in the smoking of tobacco products and alcohol consumption would have a profound influence on the incidence of oral cancer. Chemoprevention also has an impact on the development of malignant changes in the oral mucosa. Prevention through chemoprevention and/or the use of systemic medications has been an extensively studied strategy and continues to hold promise as a way of diminishing the morbidity and mortality associated with this malignancy.

Abbreviations

BBIC: Bowman-Birk inhibitor concentrate
COX: Cyclooxygenase
DMBA: 7,12-dimethylbenz(a)anthracene
EGFR: Epidermal growth factor receptor
IL: Interleukin
4-NQO: 4-nitroquinoline 1-oxide.

Conflict of Interests

The authors declared that there is no conflict of interests.

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Review Article

Techniques for Precancerous Lesion Diagnosis

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The development of the oral squamous cell carcinoma (OSCC) is a multistep process that requires the accumulation of multiple genetic alterations usually preceded by detectable mucosal changes, most often leukoplakias and erythroplakias. The clinical appearance of oral precancerous lesions and their degree of epithelium dysplasia suggests the malignization potential. Several techniques have been developed to improve the clinical and cytological diagnosis of oral precancerous lesions. The present paper reviews the main techniques used to improve premalignant lesion diagnosis.

1. Biopsy and Cytology

Oral cancer ranks as the sixth most common malignancy worldwide, 90% of which consists of squamous cell carcinoma [1–5]. Morbidity and mortality have not decreased over the past 50 years. Oral cancer early detection, mainly that of squamous cell carcinoma, is crucial to improve the patient's survival rate [1, 3–10]. The clinical diagnosis of oral precancerous lesions, leukoplakias and erythroplakias, is one of exclusion. The lesions to be excluded are those belonging to other conditions, such as lichen planus (acknowledging that it has a malignant potential itself), lupus erythematosus, leukoedema, white sponge nevus, and other lesions for which an etiology can be established, such as frictional keratosis, cheek/lip/tongue biting, contact lesions, and smoker's palate [11]. In many cases, a biopsy is mandatory so that such lesions can be discarded. Currently, histological criteria (dysplasia presence and degree) represent the gold standard in precancerous lesion risk evaluation [12].

2. Oral Exfoliative Cytology: Liquid-Based Preparations and Conventional Smears in Oral Lesions

Cytopathology is the microscopic study of cell samples collected from mucosal surfaces obtained by exfoliative cytology (via smears, scrapings, or lavage) or from internal sites via fine-needle aspiration [13]. Exfoliative cytology was first designed for cervical cancer cell early detection [14–18] and it has been primarily applied in oral medicine practice to detect early changes in oral mucosa related to malignancy. Furthermore, this exam has also been used in the diagnosis of certain types of oral lesions, most of which related to viral and fungal diseases [14].

Exfoliative cytology is performed with cytobrushes so as to obtain good-quality smear that includes cells from deeper layers of epithelium, especially of squamous intraepithelial lesions [19]. Cytological technique improvements that led to the development of a liquid-based preparation have renewed

interest in the use of this approach as an auxiliary tool in oral lesion diagnosis [14].

According to Mehrotra et al. [19], sensitivity and specificity of conventional exfoliative cytology in carcinoma's suspected lesions, ranged between 76.8%–100%, and 88.9%–100%, respectively, in a review of 22 articles. In another paper, the cytological study of oral cavity's cells was shown to be suitable for routine application in population screening programs, for early analysis of suspect lesions, and for pre-and posttreatment monitoring of confirmed malignant lesions [16].

In liquid-based cytology, the cytobrush with the sample is transported in a vial containing preservative fluid which allows the immediate fixation of cells so that all the scraped material can be used, providing high cellularity slides dispersed in a thin and homogeneous layer on a clear background, thus facilitating abnormal cell identification [14, 17, 20]. These characteristics help to establish an early oral cancer diagnosis [17, 20–23]. The use of this technique has significantly reduced the number of unsatisfactory slides, diminishing false negative results and has increased sensitivity and specificity when compared to conventional cytology [14, 21]. According to Nanove [24], the sensitivity for this technique is 95,1%, and the specificity is 99%. In another study [19], it was showed the number of inadequate samples was reduced 8,8%. However, it requires more sophisticated laboratory equipment as well as a better-trained staff to handle, process, and analyze the samples properly [20].

The cells obtained from exfoliative cytology can be used for molecular analysis. Some molecular markers may provide additional information that will be useful in malignant lesion early diagnosis. The main markers used in cytological analysis are Ki-67 [16, page 53], [19, 22], DNA ploidy status (chromosomal pairing) [18, 25], epigenetic changes (hypermethylation of the promoter region), and genomic instability, such as loss of heterozygosity instability (LOH) [22, 26] and microsatellite (MSI) [22].

3. Cytomorphometry: Computer-Assisted Analysis Brush Biopsy

Cytomorphometry, computer-assisted analysis brush biopsy (Oral CDx Laboratories, Suffern, N.Y), is a method used in the analysis of cellular samples collected by brush biopsy, a disposable specialized circular plastic brush that collects transepithelial cellular samples composed of free cells and clusters [13, 19]. The clinician rubs or rotates the brush against the lesion until pinpoint bleeding is absorbed [27]. The samples are fixed onto a glass slide and sent to a laboratory where they are stained (via a modified Papanicolaou test), scanned, and analyzed microscopically by means of a computer-based imaging system that can rank cells on the basis of their degree of abnormal morphology [13]. The analytical results and representative examples are then referred to a pathologist [19]. Results are reported as "negative" or "benign," "positive" or "atypical." Abnormal diagnoses have included "positive" (defined as definitive cellular evidence of epithelial dysplasia or carcinoma) and "atypical" (defined as abnormal epithelial changes of uncertain diagnostic significance) results [13].

After the automated analysis, the pathologist can recommend the clinical practitioner to follow further procedures (clinical control, repeated brush biopsy, surgical biopsy, etc.) [19]. Several papers have been written on this technique [19, 28–37], but few have evaluated its performance in the prevention of oral cancer [13, 30–32, 34, 38–40]. These articles have reported sensitivity values that ranged from 88% [39] to 100% [32] and 25% [38] to 96% specificity [39]. This test has been chosen to assess lesions the practitioner might not investigate further and is not recommended for the assessment of clinically suspicious lesions for which the practitioner would normally perform conventional biopsy [13, 17].

Oral brush biopsy, as a noninvasive diagnostic method, can be useful for oral mucosal lesion early detection. The occurrence of positive findings, or lesion progression despite negative findings, signals that the patient needs to be referred to a specialized clinic where a surgical biopsy should be performed, followed by histopathologycal analysis. Histopathology remains the gold standard for the definitive diagnosis of oral malignant lesions [36, 41].

4. Clinical Tissue Staining Technique

4.1. Vital Iodine Stain. Vital iodine stain (3% Lugol solution) can be used prior to biopsy and resection and is useful in the determination of the best incision area. This technique has been used in upper gastrointestinal tract endoscopy routine, as well as in cervix examination and in esophageal cancer [42]. Its principle is based on the binding of iodine to glycogen granules in the cytoplasm, resulting in a black-brown tissue color. In cancer cells, where the glycolysis is elevated [42], this method results in unstained areas whereas the normal mucosa is stained [42]. In a study with 54 patients [42], with oral squamous cell carcinoma or oral potentially malignant lesions, where the authors made surgical margins of 5–8 mm from the border of the stained lesion with vital iodine stain, it was shown that 98,1% has no recurrence after a median followup of 15 months.

4.2. Toluidine Blue Staining (TBlue Staining). Toluidine Blue (also known as tolonium chloride) is a vital metachromatic dye of the thiazine group that has been effectively used in nuclear staining because of its binding to DNA nucleus acid [2, 3, 7]. It has been used for decades as an aid in epithelium dysplasia identification [2, 10] and appears to improve precancerous lesion visualization by showing high-risk areas (areas of high cell proliferation), therefore guiding biopsy [2, 3, 7, 9, 10]. However, most studies have had problems with the absence of randomized control care and methodology [2, 3, 7, 9, 10]. The proceeding starts with a topical application of TBlue on the lesion with the aid of a swab or cotton applicator, and the more intense TBlue staining areas should be the ones elected to be biopsied. TBlue seems to be highly sensitive but has low specificity, since it also stains benign and common lesions which involve inflammation [2, 7].

TBlue is an easy and cheap technique, causes no harm to the patient, and may help to perform a careful clinical examination [3, 7]. False negative staining is very rarely observed in squamous cell carcinoma, but inflammatory lesions can contribute to false positive outcomes [7]. Some works [7, 10] have shown the sensitivity and specificity vary from 38%–98% and 9%–93%, respectively.

5. Chemiluminescence Technique

5.1. Chemiluminescence Light. The chemiluminescence technique (ViziLite (Zila Pharmaceuticals, Phoenix, Arizona)) is an exam that was approved in 2002 in the USA. It serves the purpose of improving the identification, visualization, and monitoring of oral precancerous lesions [1, 4, 8, 10, 43], and consists of the emission of light from a chemical reaction between hydrogen peroxide and acetylsalicylic acid inside a capsule light stick [4, 8, 44]. The use of a 1% acetic acid solution for washing and cleaning the oral mucosa for about 1 minute before chemiluminescence light is recommended. The action of the stick holds good for approximately 10 minutes [1, 3, 4, 44]. This reaction emits a blue/white light (430–580 nm) whose principle is based on the reflective properties of tissues that present cellular alterations such as a higher nuclear/cytoplasmatic rate. The "acetowhite" lesion is more defined and sharper, whereas the normal tissue is dark [1–4, 8–10, 44].

This seems to be an easy, safe and noninvasive system capable of helping the dentist to better visualize lesions, as well as its edges [3, 8, 44]. Another point to consider is that the lesion seems to be bigger under chemiluminescence light [44]. One disadvantage is that this system is expensive and a stick is used for each patient. Furthermore, chemiluminescence light seems to be nonspecific as it does not identify the lesion etiology—whether inflammatory, neoplastic benign, or neoplastic malign—and this could lead to unnecessary biopsies [2, 8, 44].

This system is useful for clinical examination, inasmuch as it improves the lesion visualization [9], especially when used in association with the Toluidine Blue solution to mark the lesions to posterior biopsy [2, 8]. It is known that acetic acid wash can provide a more accurate diagnosis than chemiluminescence light [2].

The ViziLite tool enhance intraoral visualization of white lesions, however it is not able to discriminate between keratotic, inflammatory, malignant, or potentially malignant oral mucosal white lesions [8, 9, 45]. The main advantage of this technique is that it significantly improves the sharpness of the lesions' margins [8, 9, 45].

6. Light Emission Technique

The Light Emission Technique (Microlux DL (AdDent, Danbury, Conn.)) seems to operate on a principle of light emission similar to that of chemiluminescence light and helps to sharpen the lesion edges as well as to improve visualization [2, 43]. In this method, the patient first needs to have a 1% acetic acid mouthwash, and then a battery-powered light source is used. An advantage of this system is that it is reusable [2, 8, 10, 43]. Another similar system uses a Led (Orascoptic DK (Orascoptic, a Kerr Company, Middleton, Wis.)) with a rechargeable battery to screen the oral mucosa and claims to improve visualization [10]. In a study [43] to assess the efficacy of acetic acid mouthwash and diffused light illumination (Microlux/DL), as a diagnostic tool in the visualization of oral mucosal potentially malignant lesions, Microlux/DL showed a sensitivity of 77.8% and a specificity of 70.7%, with a positive predictive value of 36.8%. According to the authors [43], Microlux/DL was able to enhance lesion visibility; however, it is a poor discriminator for inflammatory, traumatic, and malignant lesions.

The Narrow-emission tissue fluorescence (VELscope (LED Dental Inc. White Rock, British Columbia, Canada)) technique involves tissue exposure to different wavelengths (400 to 460 nm) in order to observe differences between normal and abnormal mucosa [2, 46]. This system involves the cell answer (autofluorescence due to cellular fluorophores) after excitation [2–4, 10]. The abnormal tissue has a different fluorophore concentration that results in changes in color [4]. This method uses a small optic fiber and consequently does not cover the entire mouth, so it is employed only for isolated lesions [2], lesion edge, and cancerization field determination [4]. While the normal mucosa glows and emits color (pale green), the abnormal mucosa shows decreased levels of fluorescence and acquires a dark magenta, brown, or black color, as it absorbs fluorescence [2, 4, 6, 47]. This technique seems to be helpful in lesion detection, but it is useless in the differentiation of malignant from benign lesions [2, 3, 6]. Despite its applicability, the system is expensive, and color interpretation is difficult, which could lead to a erroneous diagnosis [6]. Some studies in the literature [4, 6] referred this technique as having sensitivity values from 97% to 98% and specificity from 94% to 100%.

Multispectral fluorescence and reflectance (Identafi 3000 (Trimira, Houston, Texas)) is a new technique based on the tissue fluorescence principle [3, 46] which uses three types of lights: white, violet (405 nm), and amber (560 nm) [46]. According to the manufacturer, white and violet lights use the same principle as tissue reflex and fluorescence, while amber light improves vascular architecture visualization in normal and abnormal tissue [48]. Normal tissue appears defined, while the abnormal tissue has a diffuse vasculature [48]. According to the manufacturer, it is reusable [48]. This method has not been studied yet.

7. Conclusions

In this paper we approached different techniques which may be useful in the diagnosis of precancerous lesions. It is shown their applications and limitations. However, many possibilities are available, and it is concluded that the most reliable method of diagnosis is still the biopsy followed by histopathological examination.

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Review Article

Chemoprevention of Head and Neck Cancer by Green Tea Extract: EGCG—The Role of EGFR Signaling and "Lipid Raft"

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Over the past decade dose-intensified chemo-radiotherapy or molecular targeted therapy has been introduced into the treatments of head and neck squamous cell carcinoma (HNSCC) to improve the outcomes of this dismal disease. However, these strategies have revealed only limited efficacy so far. Moreover, the frequent occurrences of second primary tumor further worsen the prognosis of patients. In this context, early detection and chemoprevention appear to be a realistic and effective method to improve the prognosis as well as quality of life in patients with HNSCC. In this short paper, we discuss the potential of green tea extract, (-)-epigallocatechin-3-galate (EGCG) in HNSCC chemoprevention, focusing on two aspects that are provided recently: (1) evidence of clinical efficacy and (2) unique biological effects on "lipid raft" that emerged as an important platform of numerous biophysical functions, for example, receptor tyrosin kinases (RTKs) signalings including epidermal growth factor receptor (EGFR), which play critical roles in HNSCC carcinogenesis.

1. Introduction

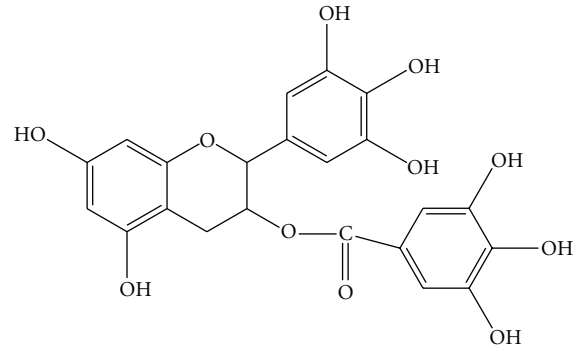
Head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer worldwide, often generates from critical organs including the larynx, pharynx, oral cavity, and tongue that play indispensable roles in social, respiratory, communicative, and nutritional functions [1]. Surgical intervention for these organs often leads to a considerable impairment of the patient's quality of life (QOL), albeit recent remarkable progresses in reconstructive surgery. Accordingly, the intensity of conventional DNA-damaging therapies (i.e., irradiation and chemotherapy) has been strengthened to the upper limit of human tolerance of acute toxicities during the last decade [2]. Short-term results of these treatments seem to be promising. However, it is still under debate whether these dose-intensified types of protocols would lead to the long-term overall survival as well as "functional" organ preservation, because these protocols occasionally cause considerable complications (e.g., requirement for feeding tube due to severe laryngeal and pharyngeal dysfunction) and potential treatment-related death [2–4]. Ongoing molecular targeted therapies in HNSCC revealed only marginal effects so far [5]. In addition, the frequent occurrences of second primary tumor further worsen the prognosis of patients with HNSCC [1, 6]. As a result, the dishonorable phrase that is routinely used in the *Introduction* of HNSCC studies: "Despite recent advancements in treatment modalities, the overall survival and QOL of patients with HNSCC have not improved significantly over the past decade" still holds true, especially for patients with advanced stage. In view of these findings, early detection and chemoprevention appear to be realistic and effective method to improve the prognosis as well as QOL of patients with HNSCC.

2. Evidence and Perspective of EGCG in Chemoprevention

As indicated by a recent review, we have witnessed remarkable progresses in the chemoprevention research in HNSCC [6]. A variety of natural and synthetic compounds have been shown to exert chemopreventive effects on HNSCC. Among them, a major active component of green tea extract, (-)-epigallocatechin-3-galate (EGCG), seems to be one of the most promising compounds that displays tumor suppressive effects on animal carcinogenesis model, mouse xenograft model, and a variety of cancer cell lines [7]. Figure 1 demonstrates the chemical structure of EGCG. Despite these substantial experimental data, there has been a longstanding question about the clinical efficacy of EGCG, because in a majority of *in vitro* studies, EGCG exhibits biological functions at relatively higher concentrations compared to the peak plasma concentrations obtained in individuals after administrating an oral dose of EGCG or decaffeinated green tea extract (<1 μM) [8, 9]. However, recent studies provided evidence that administration of EGCG indeed has potential to reverse the process of carcinogenesis in patients with HNSCC or other human malignancies. In a phase II trial, Tsao et al. examined the effects of administration of green tea extract (GTE) capsule that contains 13.2% of EGCG for 12 weeks, three times a day at the dosage of 0 (placebo), 500, 750, or 1000 mg/m^2/day on 41 patients with high-risk oral premalignant lesion. They found that two high dose arms (750 and 1000 mg/m^2) revealed significantly ($P = .03$) higher response rates (58.8%) than 500 mg/m^2 (36.4%) or placebo (18.2%) [10]. The group of Shimizu, who is one of the authors of this paper, demonstrated that administration of 500 mg of GTE tablet that contain 52.5 mg of EGCG three times a day (total 1500 mg/day) for 12 months significantly ($P < .05$) inhibited the incidence of second metachronous colon adenoma in patients who underwent endoscopic polypectomy, thus 31% in control arm versus 15% in the GTE treated arm [11]. Patients with high-grade prostate intraepithelial neoplasia received either placebo or 200 mg of GTE capsule that contain 51.8% of EGCG three times a day (total 600 mg/day) for 12 months. The GTE group displayed significantly ($P < .01$) lower incidence (3.0%) of prostate cancer compared to the placebo group (30.0%) [12]. No serious adverse effects were observed in any of these trials. Collectively, these studies indicate that administration of 50–200 mg of EGCG three times a day for 12 months appears to be safe and clinically effective protocol. Thus, the setting appears to be ideal for validating the clinical efficacy of EGCG in a larger-scale chemoprevention study.

3. Diverse Molecular Target of EGCG

A rapidly increasing number of mechanistic studies have revealed that in addition to the antioxidant effect, EGCG inhibits tumor development and progression by modulating wide spectrum of molecular targets. Those include RTKs: epidermal growth factor receptor (EGFR), erbB2/Her2, erbB3/Her3, erbB4/Her4, vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGR), insulin-like growth factor receptor (IRGFR) and hepatocellular growth factor receptor (HGFR), mitogen activated protein kinase (MAPK), proteasomes, matrix metro proteases (MMPs), cyclin-dependent kinases (CDKs), p53, DNA methyltransferase, Bcl-2, VEGF, reactive oxygen species (ROS,) 67 kDa laminin receptor (67LR), vimentin, phosphatidylinositol-3-kinase (PI3K)-Akt, NF-κB, signal transducers and activators of transcription 3 (Stat3), and AP-1. These surprisingly diverse interactions between EGCG and target molecules or pathways are summarized in a recent comprehensive review [7]. In this short paper, we will mainly discuss the effects of EGCG on receptor tyrosin kinases (RTKs), especially EGFR, and their cell surface vessel, "lipid rafts," that have emerged as a critical target of EGCG as well as an essential platform for signal transduction.



(−)−epigallocatechin-3-gallate (EGCG)

Figure 1: Structure of EGCG.

4. The Role and Mechanism of EGFR Activation in HNSCC Carcinogenesis

In 1990s, Grandis et al. demonstrated that EGFR and its ligand transforming growth factor-α (TGF-α) mRNA were overexpressed in approximately 90% of HNSCC tumors, and overexpression of these two proteins was significantly associated with poor prognosis of patients with HNSCC [13, 14]. To date, numerous studies have revealed that EGFR signaling orchestrates tumor development and progression by activating several downstream signal transduction pathways including MAPK, Stat3, PI3K-Akt-mTOR, protein kinase C (PKC), and NF-κB [15–17]. Several mechanisms have been postulated to explain aberrant EGFR signaling in human malignancies [15, 16]. Those include (1) receptor overexpression, (2) autocrine or paracrine activation by ligand overexpression or excessive ligand cleavage from cell surface by ADAM family metalloprotease, (3) gene amplification, (4) ligand independent activation through other receptor systems (e.g., erbB2), (5) constitutive active EGFR mutants: somatic activating mutation or truncated EGFRvIII, and/or (6) loss of negative regulation (e.g., EGFR degradation). Despite EGFR is one of the most extensively investigated molecules in HNSCC pathogenesis,

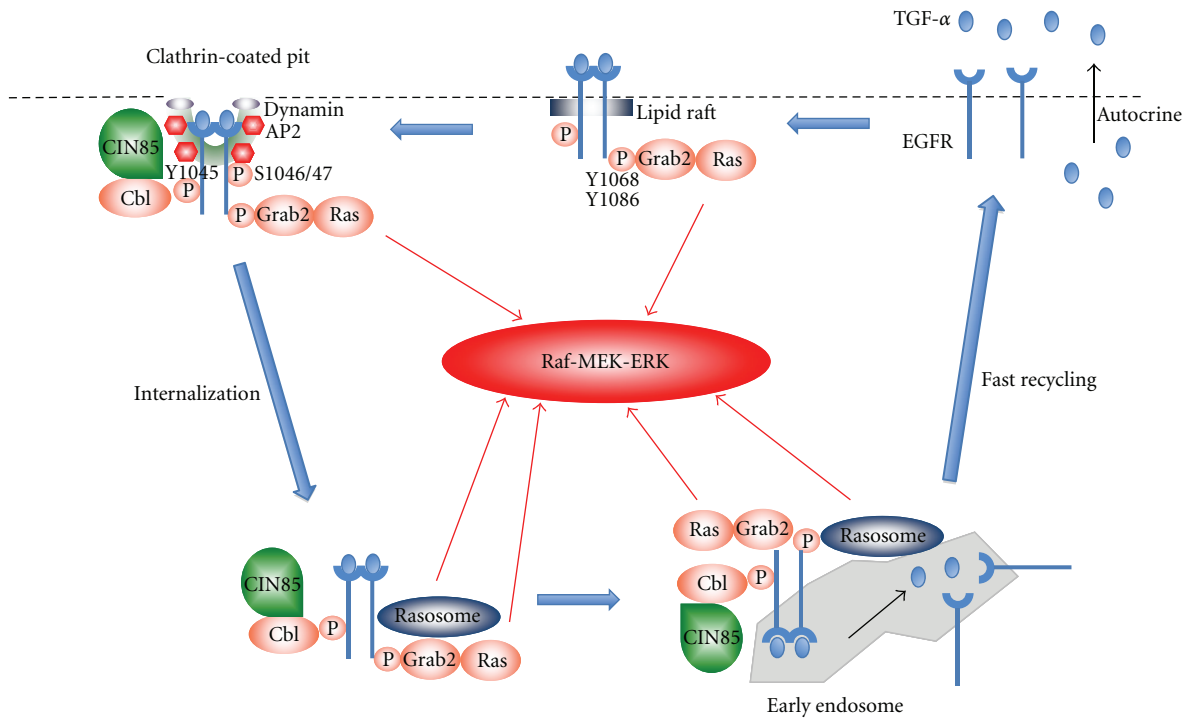


Figure 2: A proposed mechanism of TGF-α/EGFR/ras-MAPK activation loop in HNSCC. TGF-α binding to EGFR leads to dimerization and phosphorylation on lipid rafts. Phosphorylation of Y1068 and Y1086 is required for Grab2 binding and consequent ras activation. Activated EGFR dimmer is internalized via clathrin-coated pit. Cbl and CIN85 (overexpressed in 40% of HNSCC samples) are recruited at pY1045 and facilitate EGFR internalization. Phosphorylation of S1046/1047 is also necessary for EGFR internalization, albeit the precise role remains elusive. Recent evidence suggests that TGF-α-bound EGFRs signal in the cytosol, activating ras-MAPK cascade. Ras-enriched small cytosolic nanoparticles, "rasosomes," might contribute to this signaling. Internalized TGF-α-bound EGFRs are sorted to early endosome. TGF-α dissociates from EGFR in the acidic environment of endosome. Free EGFR is recycled back via fast recycling back pathway to plasma membrane and is activated by TGF-α in a autocrine manner, resulting in constitutive activation of TGF-α/EGFR/ras-MAPK.

the predominant mechanism of EGFR activation remains elusive. The EGFR gene amplification was observed only in 7 out of 33 patients with HNSCC, and intriguingly this amplification did not lead to protein overexpression [18]. However a recent study demonstrated that 49 out of 145 oral premalignant lesions displayed EGFR protein overexpression which was associated with relatively high incidence (41%) of EGFR gene copy [19]. Thus, the correlation between the EGFR gene amplification and protein expression is still under debate. The possibility of excessive cleavage of TGF-α and amphiregulin was demonstrated in HNSCC cell lines [20] but is not confirmed in clinical samples. The reported rates of somatic mutation of EGFR in HNSCC range as low as 7-8% [21–23]. Sok et al. found EGFRvIII expression in 42% of 33 HNSCC samples employing both immunohistochemical and RT-PCR assays [24]. In contrast, Yang et al. reported only 15% of EGFRvIII positive rates in 39 Chinese laryngeal cancer [25]. Interestingly, in 82 HNSCC samples from Japanese population, EGFR vIII was not detected [23]. Here again, the role of EGFRvIII in HNSCC is still controversial. The mechanism of EGFR internalization, degradation and recycling is a quite essential aspect that is closely associated with EGFR signaling [26]. However, there were few reports, which investigated this mechanism in HNSCC. We recently examined the role of multiadaptor protein c-Cbl interacting protein of 85 kDa (CIN85) [27] in HNSCC focusing on its role in EGFR signaling pathway [28]. In this study, we found that (1) CIN85 significantly facilitates EGFR internalization without apparently altering the levels of phosphorylated EGFR protein (i.e., EGFR signal intensity), consistent with the theory that TGF-α bound EGFRs are mainly sorted to the recycling-back pathway escaping from degradation, while a majority of EGF-bound EGFRs are processed via the degradation pathway [26], (2) TGF-α bound EGFR receptor signals in the cytosol as well as on plasma membrane, activating ras-MAPK cascade (ras-enriched small cytosolic nanoparticles, "rasosomes," might contribute to this signaling [29]), (3) CIN85 silencing, therefore, inhibits EGFR internalization and activation of ras-MAPK cascade, and (4) CIB85 overexpression observed in 40% of HNSCC tumor samples contributes to the development of EGFR/ras-MAPK activation loop (Figures 2 and 3(a)). This model, at least in part, accounts for the reason why not EGFR but TGF-α is prominent mitogen in HNSCC development and progression. Nevertheless, it should be emphasized that the mechanism that causes TGF-α and EGFR overexpression in HNSCC remains elusive, although almost 20 years have passed since Grandis et al. [13, 14] first reported the significance of this overexpression.

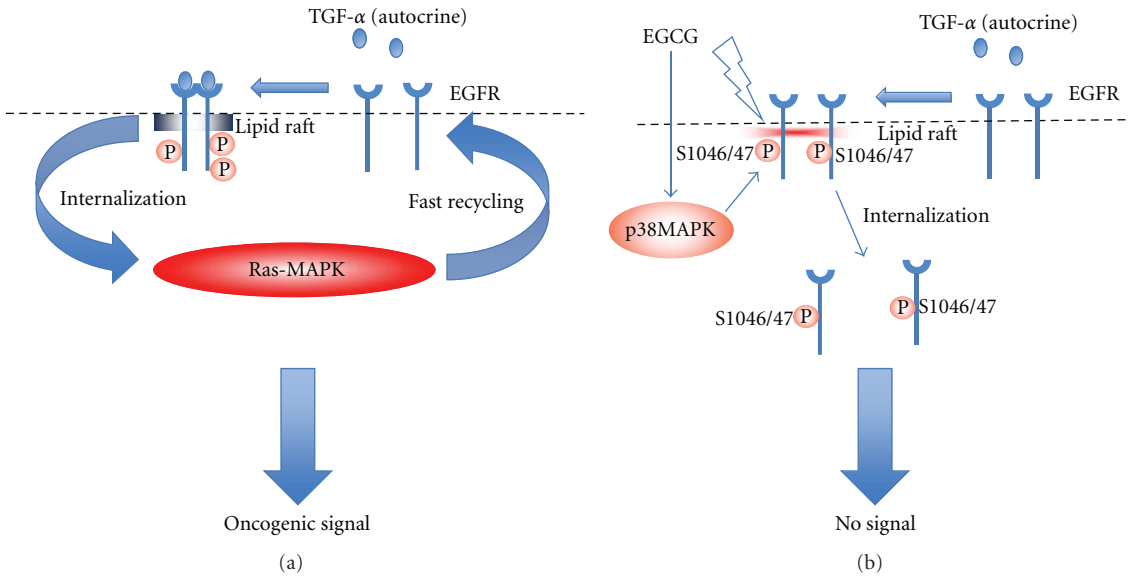


Figure 3: (a) Summary of TGF-α/EGFR/ras-MAPK activation loop in HNSCC (for more detail see Figure 2). (b) Inhibitory effect of EGCG on EGFR activation. EGCG alters organization of lipid rafts and promotes internalization of nonactivated monomer EGFR into cytosol through phosphorylation of EGFR at serine 1046/1047 by p38MAPK. As a result, EGCG causes a marked reduction of phosphorylated EGFR and thereby inhibits EGFR signaling that is prominent in HNSCC.

5. EGCG Inhibits EGFR: The Role of Lipid Raft

Irrespective of the mechanisms which underlie EGFR activation in HNSCC, it was discovered by Liang et al. that EGCG can directly inhibit the binding of EGF to EGFR and thereby inhibits EGFR signaling [30]. Consistent with this finding, we first provided evidence that EGCG indeed inhibits EGFR activation in HNSCC cell line that displays autocrine activation of EGFR by TGF-α [31]. We further examined the effect of EGCG on erbB2/Her2 employing HNSCC and breast cancer cell lines, and found that EGCG can inhibit the erbB2/Her2 activation, demonstrating the first example of erbB2/Her2 inhibition by EGCG in human malignancies [32]. Thereafter, we and other investigators confirmed the inhibitory effects of EGCG on other RTKs including erbB3/Her3, erbB4/Her4, IGFR, PDGFR, FGFR, and VEGFR employing a variety of cancer cell lines derived from different organs [33–38]. These ubiquitous inhibitory effects of EGCG on a series of RTKs, combined with the fact that the inhibitory effect of EGCG on EGF/EGFR binding was found only in a subcellular system [30], raised a question that EGCG might inhibit RTKs by a more general mechanism.

Due to recent remarkable progresses in methods to analyze the structure, dynamic assembly, and function of nanoscale molecules, it is beginning apparent that cell membranes play critical roles in coordinating a variety of biochemical reactions including RTKs signal transduction [39–42]. Nanoscale transient membrane domains, "lipid rafts," that are enriched with cholesterol, glycosphingolipids, glycosylphosphatidylinositol-anchored protein, caveolin-1, and signaling molecules, function as signaling platforms [39–41]. Among the RTKs, the interaction of EGFR with lipid rafts is most well understood [39]. Activation of EGFR by ligand and consequent signal transduction begins at lipid rafts, while its internalization occurs at clathrin-coated pit by further recruiting the E3 ubiquitin ligase Cbl, CIN85, and endorphins. The role of CIN85 in EGFR signal transduction in HNSCC was discussed in the previous section. These observations made us to hypothesize that EGCG might inhibit the activation of EGFR or other RTKs by altering the formation of lipid rafts.

So far through a series of three studies [43–45] we found that (1) EGCG alters lipid organization on the plasma membrane, (2) EGCG promote the internalization of nonactivated monomer EGFR into cytosol, thus, inhibiting the activation of EGFR by EGF, (3) as a result, treatment with EGCG causes marked reduction of phosphorylated (activated) EGFRs, that are otherwise preferentially present in lipid rafts, (4) EGCG-induced EGFR internalization requires neither the binding of c-Cbl to EGFR nor a phosphorylation of EGFR at tyrosine residue, suggesting that this internalization is mediated by a different mechanism that is observed in EGF-treated cells, and (5) phosphorylation of EGFR at serine1046/1047 mediated by p38MAPK is essential for EGCG-induced EGFR internalization (Figure 3(b)).

In parallel with our findings, a Japanese research group discovered that 67LR, a constituent protein of lipid rafts, is an important binding target of EGCG [46]. 67LR is a nonintegrin laminin receptor, which is overexpressed on cell surface of various types of tumors, and the expression level of this protein strongly correlates with the aggressive phenotypes of tumor, albeit its role in HNSCC carcinogenesis is

not investigated so far [47, 48]. Intriguingly, the predicted K_d value for the binding of EGFG to 67LR is as low as 40 nM, and physiological concentration of EGCG indeed inhibits the growth of human lung cancer cell line in a 67LR-dependent manner [46]. Although it is not clear whether the above-mentioned inhibition of EGFR by EGCG is relevant to 67LR, this finding also provides evidence that EGCG exerts antitumor effects through the interaction with lipid rafts protein.

As mentioned in the "*Introduction*," the EGFR targeted therapies, either used alone or in combination with radiation, have shown only limited efficacy so far, albeit its significant role in HNSCC carcinogenesis [5]. One of possible explanations for this insensitivity is that other growth factors or cytokines can surrogate EGFR signaling and activate downstream signal cascades including MAPK, Stat3, and PI3k-Akt. Then, HNSCC can relatively easily escape from EGFR dependency. However, Zhang et al. demonstrated that EGCG can synergistically enhance the growth inhibitory effects of EGFR tyrosine kinase inhibitor, erlotinib, both *in vitro* and in animal xenograft models employing HNSCC cell lines [49]. Consistent with our findings, treatment of EGCG significantly enhanced EGFR internalization that was not observed with treatment of erlotinib alone. Thus, they speculate that this internalization and consequent degradation of EGFR might be a major mechanism that accounts for this synergistic interaction. However, given the fact that a majority of growth factors or cytokines, which can surrogate EGFR signaling, utilize lipid rafts as signaling platforms [48], this synergistic interaction might be caused through the general inhibitory effects of EGCG on these growth factors or cytokines in lipid rafts. Thus, the addition of EGCG to RTKs targeting therapies might be an attractive strategy, which leads to the prevention of drug-tolerance, as is frequently observed in several clinical settings.

6. Conclusions

Considering the tantalizingly marginal improvement in the treatment outcomes of patients with HNSCC, it is urgent and critical to develop novel strategy based on early detection and chemoprevention. Among numerous putative chemopreventive agents, EGCG appears to be one of the most promising natural compounds based on accumulated data and, in particular, two novel findings provided recently: (1) clinical efficacy and (2) unique biological effects on lipid rafts that are an important platform of numerous biophysical functions including RTKs signalings. A larger-scale clinical study of EGCG is highly recommended.

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Conflict of Interests

The authors disclose no conflict of interests.

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Review Article

Impact of HPV in Oropharyngeal Cancer

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The incidence of oropharyngeal cancers has increased in the western world and Human Papilloma Virus (HPV) has been recognised as a risk factor in the last decades. During the same period the prevalence of HPV in oropharyngeal tumours has increased and HPV has been suggested responsible for the increase. The HPV-positive tumours are today recognized as a distinct subset of head and neck cancers with its own clinopathological and risk profile and have a significantly improved prognosis regardless of treatment strategy. This review summarizes current knowledge regarding human papillomavirus biology, oncogenic mechanisms, risk factors, and impact of treatment.

1. Introduction

Head and neck squamous cell carcinoma is the sixth most common cancer worldwide [1]. The incidence of head neck cancers varies widely around the world and even within populations. Oral and oropharyngeal cancer constitutes 3–5% of the malignancies in Europe, while this figure in parts of Southeast Asia and India reaches up to 40–50% [2–4]. Eighty to ninety percent of head and neck cancer cases are considered to be associated with known risk factors, such as smoking, betel nut chewing, and alcohol abuse [4, 5].

The prognosis for HNSCC is generally low in the more advanced stages and there has been only a modest improvement in recent years and the treatment frequently sentences the patient to life-long sequelaes such as difficulties with swallowing, dryness of the mouth, esophageal strictures, and osteoradionecrosis. Head and neck cancer is a heterogeneous group that differs greatly in tumour aggressiveness and response to treatment. The treatment is today based on tumour stage which thus leads to suboptimal outcome for some patients. The identification of predictive markers is urgent to enable optimization of treatment and reduction of sequalae for the individual patient.

Despite a decreasing incidence of head and neck squamous cell carcinoma in general, attributed to a decrease in the prevalence of smoking [6], the incidence of oropharyngeal SCC is rising [7–12]. Human papillomavirus (HPV) has for some time been suggested to be involved in the carcinogenesis of oropharyngeal cancer. The Agency for Research on Cancer (IARC) now recognizes HPV as a risk factor for oropharyngeal cancer, and accumulating molecular and epidemiological data now show that high-risk types of HPV are responsible for a subset of oropahryngeal cancer [13–15]. HPV-positive cancers cases are now in majority in the western world and these tumours are also shown to have better outcome than the HPV-negative patients. However, the natural history and the tumour development biology of HPV-infection in head and neck tumours are not yet fully understood and the best management needs further investigation and clinical trials in order to achieve the best clinical outcome for the patients with HPV-positive head and neck tumours.

2. Human Papilloma Virus (HPV) and Tumour Biology

HPV was first identified in 1949 [16] and today over 100 different HPV types have been characterized [17]. HPVs are small, circular double stranded DNA viruses with a genome that consists approximately 8000 base pairs. HPV infection

is highly restricted to basal cells in the mucosal or epithelial layers. Replication occurs within the nucleus of the infected cell and is dependent on S-phase entry since it requires the DNA machinery [18]. The HPV subtypes are divided into high-risk and low-risk HPV regarding their malignant potential. Approximately 15 high-risk subtypes are known but only HPV subtypes 16, 18, 31, 33, and 35 have been identified playing a role in the development of oropharyngeal head and neck cancer. HPV 16 is the far most common type detected in oropharyngeal cancer accounting for 90–95% of the HPV positive tumours [19].

High-risk HPVs produce 2 oncoproteins, E6 and E7, which are necessary for viral replication through their proliferation stimulating activity and play a key role in malignant transformation and maintenance. The E6 oncoprotein binds and induces the degradation of the p53 tumour suppressor protein via an ubiqutin-mediated process disrupting the p53 pathway which leads to uncontrolled cell cycle progression [20, 21]. The HPV E7 protein binds and degrades the retinoblastoma protein (pRb), preventing it from inhibiting the transcription factor E2F resulting in loss of cell cycle control. Furthermore, the functional inactivation of Rb results in upregulation of the p16-protein. P16 is encoded by the CDKN2A tumour suppressor gene and regulates the activity of CyklinD-CDK4/6 complexes that phosphorylate Rb leading to release of the transcription factor E2F which initiates cell cycle progression. The bound Rb-E2F protein acts as a negative regulator by inhibiting transcription CDKN2A, and therefore the functional inactivation of Rb by E7 thereby the transcriptional inhibition of the p16 gene is lost. HPV-positive tumours are consequently characterised by high expression of high levels of p16 [22]. p16 protein can be detected by technically simple immunohistochemistry, and since several studies have shown a very high correlation (>90%) to HPV-positively in oropharyngeal tumours, it has been suggested as a clinically useful sorrogatemarker [23, 24].

In head and neck cancer caused by the traditional risk factors, tobacco and alcohol, p53 is commonly mutated [25, 26] and 9p21-22 is lost early in carcinogenesis resulting in the loss of the tumour suppressing gene p16 [27]. In contrast, HPV-positive head and neck tumours have decreased expression of wild-type p53 due to the inactivation and degradation by the E6 oncoprotein. Furthermore, in a study by Westra et al. in 2008 [28] it was shown that HPV 16 and mutated 53 may coexist in a subset of head and neck squamous cell carcinoma but HPV 16 and disruptive p53 mutations seemed to be nonoverlapping events. An inverse relationship between HPV-16 infection and disrupted p53 gene mutations in head and neck carcinomas was suggested, and thus HPV positive head and neck tumours represent a distinct molecular phenotype with a unique mechanism of tumorigenesis independent of the mutagenic effect of tobacco and alcohol.

3. Risk Factors for HPV

In contrast to the HPV-negative cancers in the head neck region the vast majority of HPV-positive cancers lack association with the traditional risk factors, tobacco and alcohol [29]. Epidemiological studies on HPV-associated cervical cancer have clearly demonstrated that HPVs are transmitted by sexual contact [30] and today there are several studies suggesting that also HPV-positive head and neck tumours are sexually transmitted. It is assumed that HPV infection precedes the development of HPV positive head and neck cancers, and the presence of high-risk HPV infection on the oral mucosa and seropositivity increase the risk of development of head and neck cancers [13, 14, 31, 32]. Therefore risk factors for HPV oral infection are likely to be risk factors for HPV-positve head neck tumours. Oral HPV infections are rare in newborns of HPV-infected mothers and in children prior to sexual activity; infections increase after onset of sexual activity. In addition, an increased risk for tonsillar cancer among women with cervical lesions and a higher rate of tonsillar and tongue cancers among husbands of women with cervical dysplasia or cancer have been identified [33]. The risk of HPV-positive head and neck tumours has been associated with sexual behaviour including increasing numbers of both vaginal and oral sex partners, young age at first intercourse, and history of genital warts [34–37]. Also other life style factors like poor oral hygiene and marijuana use have been discussed and the most recent report found that the risk of developing an HPV-16 positive head and neck cancer increased with increased marijuana use but no correlation to oral hygiene was found [37]. Whether tobacco and alcohol increase the risk is not clear where some studies found no association to development of HPV-positive head neck cancers [13, 37] while others have found that thier use increase the risk [38]. Patients with the Fanconi anaemia have increased risk of HPV-mediated tumourigenesis [13] and some data indicate that HIV-patients have increased rates of HPV-related disease in the oral cavity despite antiretroviral therapy [39, 40].

4. Epidemiology

As for other head and neck cancers, the incidence of orophayrngeal cancer rates varies widely around the world and even within population significant differences have been observed. The black population in the United States, for instance, tends to have higher incidence rates than whites and hispanic populations all over the country. The SEER, Surveillance Epidemiology and End Results, covers approximately 14% of the US population and the age-standardized rates of tonsillar cancer in whites were 1.4 for men and 0.4 for women in 1993–1997. For blacks the rates were 2.9 and 0.6, per 100 000 person-years, respectively.

By contrast, in China, the rates of tonsillar cancer are generally low, for instance, in Beijing where the rates were 0.1 for men and 0.0 for women, respectively. Interestingly, in Hong Kong and in Taiwan, places with great western influence, the rates were 6 to 12 times higher than in Beijing. In India, with high rates of oral cancer, the rates of tonsillar cancer were between 0.8 and 2.8 in men and 0.2 and 0.5 in women, respectively.

In most countries the rates tend to be higher in males than in females with a ratio from 2 : 1 to 5 : 1. However, in the Philippines and in Vietnam women had higher incidence rates than men. Interestingly, this was true also for the Philippine population in California.

Also in Europe, the incidence rates show great variations with intranational variability in some countries. The highest rates were seen in parts of France, in Somme, where the rates were as high as 6.4 for men and 0.8 for women [41].

In several western countries, the incidence of oropharyngeal cancer has increased greatly in the last decades [7–9, 42–44] and the incidence has increased most in men. At the same time, the prevalence of HPV in those tumours has increased in a similar way indicating that HPV in fact is responsible for this increase [11, 42, 45]. HPV has been found in 45–95% of the oropharyngeal tumours [11, 42, 46] and the prevalence of HPV 16 has been quite homogenous around the world [47] in contrast to cervical cancer, where the prevalence of different types of HPV varies around the world [48].

5. HPV in Head-Neck Cancers

Apart from having a different epidemiology and aetiology, the HPV-positive oropharyngeal cancers also constitute a distinct subgroup clinopathologically. HPV-positive tumours are usually poorly differentiated and nonkeratinizing and have a basaloid appearance in contrast to the HPV-negative that is more moderately differentiated and keratinizing [49, 50]. HPV-positive tumours also demonstrate significantly lower levels of chromosomal mutations than the HPV-negative tumours [51, 52]. Furthermore, patients with HPV-positive oropharyngeal cancers in general, especially tonsillar cancers, tend to be younger at time of diagnosis [42, 53], possibly with the exception of base of tongue cancers where no age difference could be found between the HPV-positive and HPV-negative cancer patients [10]. The majority of the patients have no prior history of tobacco and/or high alcohol consumption and have generally a better performance status compared to the HPV-negative patients [37, 54]. Moreover, HPV-postive tumours often present at a higher stage with a small T-size (T1-T2) [55] but frequently there is a large, often cystic, nodal involvement (N+) [56, 57], thus the HPV-positive tumours are often diagnosed in clinical advanced stages, that is, Stages III-IV [15].

6. Clinical Implications/Effect on Prognosis

The prognosis for HPV-positive oropharyngeal cancer patients is better than that for patients with HPV negative tumours independent of nodal status, age, stage, tumour differentiation, or gender [1, 15, 58–60]. Several studies have shown 80–95% 2-3 years overall survival rate for the HPV-positive patients compared to 57–62% for the HPV-negative subgroup of oropharyngeal tumours [60–62].

While it is still unclear whether tobacco is a risk factor for HPV-induced oropharyngeal tumors, it seems clear that smoking has a negative impact on relapse and survival for the HPV-positive tumours [63]. The risk of death and cancer relapse significantly increased by 1% for each additional pack year of tobacco smoking [61], indicating that the biological behaviour of an HPV-positive tumour may be altered by tobacco use.

Treatment of head and neck tumours today is often standardized and based on tumour stage despite the knowledge of the heterogeneity regarding tumour aggressiveness and response to treatment of the tumours varies greatly. Treatment of patients with advanced disease often includes both oncological and surgical treatment as both carry acute side effects and lifelong sequelae. The surgical trend in the last years, especially regarding neck dissection, has turned from a very radical operation towards more organ preserving selective/modified neck dissections when possible, reducing the morbidity. In contrast, the oncological treatment has turned in the opposite direction including the development of altered fractionation radiotherapy, integration of chemotherapy with radiotherapy, incorporation of intensity-modulated radiotherapy, and the introduction of targeted biological therapy. The combined modality treatment and the altered/intensified fractionation have improved outcome for head neck cancer patients in general [64, 65], but the morbidity has also significantly increased [66–68]. However, today there is no absolute consensus about patient selection for altered fractionation regimens, type of chemo-radiotherapy association, radiation, or chemotherapy dose schedule. Among older patients with advanced disease, age over 70 years, the compliance to treatment is low due to significant comorbidity and poor performance [68]; thus this intense therapy is probably not suitable for this group of patients.

Patients with HPV-positive tumours in the oropharynx show an improved survival regardless of treatment strategy. Superior outcome for HPV-positive tumours has been shown for surgery [69], convectional and modified fractionated radiotherapy [55], induction chemotherapy [60], concurrent chemotherapy [61] and induction chemotherapy plus concurrent chemotherapy [60]. Thus, regarding patients with HPV-positive tumours in oropharynx the debate today is whether the intense therapy is too aggressive in this group of patients since they show a superior survival regardless of treatment strategies.

Strengthening this theory, a recent published study by Ang et al. [61] showed no significant difference in overall survival between a concomitant boost accelerated fraction regimen of radiotherapy and a standard fractionation regimen when combined with concurrent high-dose cisplatin for HPV-positive patients.

The reason for the better response to treatment for patients with HPV positive tumours is not known. There are studies that have found an inverse relationship between tumour HPV status and presence of p53 mutations in head and neck cancer [50, 70]. The improved response to oncological treatment observed for patients with HPV-positive tumours could therefore be explained by the presence of an intact p53-mediated apoptotic response in HPV-positive tumours. Another possibility is immunological factors related to HPV infection [71].

Today, there is a subgroup of the HPV-positive oropharyngeal cancers that have worse clinical outcome, that do not respond as well to given treatment, and have a higher rate of relapses and worse survival than the majority of the tumours in this group. A question for the future is how to separate the HPV-positive oropharyngeal tumours in this relative small group. Several potential clinical markers have been suggested, p16 [23, 24, 55, 72], Cyklin D1, [73], EGFR [74–76], p53 [28], and p21 [73], and maybe a combination of these and other not yet known markers may provide additional prognostic information and thus guide us to select the right patients for the right combination of treatment.

Based on the profound impact of HPV on the response to treatment for patients with oropharyngeal tumours, HPV-status should be included to standard pathological reporting in clinic and the planning of treatment strategies for this group of patients. Future clinical trials comprising patients with oropharyngeal tumours should include HPV status as a stratification factor in order to identify the least morbid treatment to cure this group of patients.

7. Concluding Remarks

HPV-positive oropharyngeal cancer is recognized as a distinct subset of head and neck squamous cell carcinoma with a favourable outcome. HPV status is a profound prognostic factor for overall and progression-free survival, treatment response, and tumour control. The superior outcome is independent of nodal status, age, stage, tumour differentiation, or gender and regardless of treatment strategy. The explanation for this difference is not clearly understood today and is probably a combination of several factors, patient-related (younger age, less exposed to tobacco and alcohol, less co morbidity, etc.) as well as tumour-related factors (presence of p53-mediated apoptosis).

HPV-positive oropharyngeal tumours represent a distinct clinopathological profile where there still exist many questions to be answered regarding tumour biology and how the disease develops (tumouregenesis).

In future, clinical trials on oropharyngeal tumours need to take HPV-status in consideration. There is a possibility that less intense treatment strategies with lower rate of acute and long-term side effects do not compromise the survival outcome for this group of patients. Since patients with HPV-positive oropharyngeal cancers often are younger and in good health, few severe life-long complications are very important considerations. We need further knowledge about the tumour biology and identification of additional clinical useful markers to combine with HPV-status for appropriate risk stratification in future clinical trials in order to optimize the treatment for each individual patient in the future.

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Review Article

Role of Brush Biopsy and DNA Cytometry for Prevention, Diagnosis, Therapy, and Followup Care of Oral Cancer

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Late diagnosis resulting in late treatment and locoregional failure after surgery are the main causes of death in patients with oral squamous cell carcinomas (SCCs). Actually, exfoliative cytology is increasingly used for early detection of oral cancer and has been the subject of intense research over the last five years. Significant advances have been made both in relation to screening and evaluation of precursor lesions. As this noninvasive procedure is well tolerated by patients, more lesions may be screened and thus more oral cancers may be found in early, curable stages. Moreover, the additional use of DNA image cytometry is a reasonable tool for the assessment of the resection margins of SCC. DNA image cytometry could help to find the appropriate treatment option for the patients. Finally, diagnostic DNA image cytometry is an accurate method and has internationally been standardized. In conclusion, DNA image cytometry has increasing impact on the prevention, diagnostic, and therapeutical considerations in head and neck SCC.

1. Introduction

Patients with squamous cell carcinomas of the oral cavity have a fair prognosis with an overall 5-year survival rate of about 45% [1]. Unfortunately, this figure has not substantially improved during the past 30 years [2]. Late diagnosis resulting in late treatment and locoregional failure after surgery or even after combined surgery and radiotherapy are the main causes of death in patients with oral squamous cell carcinomas.

These days, an alternative method for the examination of oral lesions is exfoliative cytology. It is based on the technique of Papanicolaou, which is accepted worldwide, as a successful method in order to screen for epithelial dysplasias in situ or invasive carcinomas of the uteri cervix. Currently, exfoliative cytology is increasingly used for early detection of oral cancer and has been the subject of intense research over the last five years [3, 4]. Significant advances have been made both in relation to screening and in the evaluation of precursor lesions [5–11]. Although mucosal biopsy is still regarded as the gold standard for definitive oral cancer diagnosis, exfoliative cytology is a valuable tool for the noninvasive evaluation of a range of potentially preneoplastic oral mucosa lesions, like leuko-/erythroplakias and lichen ruber. The cytometric detection of DNA aneuploidy in exfoliated suspicious respectively dysplastic cells, qualifies these as malignant, up to two years earlier than cytology or histology alone [12, 13].

2. Prevention

2.1. Precursor Lesions of Oral Cancer. Oral carcinogenesis proceeds through a stepwise accumulation of (cyto)genetic changes over time. Because the oral cavity is easy to examine and risk factors for oral cancer are known, there is great opportunity to improve patient outcomes through diagnosis and treatment of premalignant lesions before the

development of invasive oral carcinoma [14]. In contrast to the oral premalignant *conditions*, oral premalignant *lesions* are morphologically abnormal solitary or multiple areas of mucosa that are typically white, red, speckled or verrucous in appearance. The WHO classification [15] combines leukoplakia and erythroplakia into "precursor lesions," with a 6.8% estimated rate of transformation of oral leukoplakias to cancer. It identifies proliferative verrucous leukoplakia as a separate high risk lesion with minimal cytological atypia. Oral lichen planus, a chronic inflammatory condition, also is associated with an increased risk of cancer development of about 3% [16, 17].

2.2. Indications for Brush Biopsy. Screening for oral cancer and its precursor lesions may be performed by dentists, dental surgeons, and other health care professionals. Exfoliative cytology, taking brush biopsies, is advocated for evaluation of macroscopically suspicious lesions of the oral mucosa that are detected clinically by screening. This may be followed by a mucosal scalpel biopsy. Yet, exfoliative cytology may replace tissue biopsy in lesions that are clinically not obviously suspicious for malignancy but nevertheless need surveillance. As tissue biopsy is associated with lower compliance by patients as compared to brush biopsy, this noninvasive approach may lead to a higher number of investigated suspicious oral lesions and thus to an increased rate of curable cancers, identified in early stages.

3. Sampling of Cells

Collection devices suitable to obtain cells from the superficial and intermediate layers of the oral mucosa may be conventional brushes, as used for endocervical sampling by gynecologists, such as the CytoBrush and Orca Brush (Figure 1). The brush is rotated under slight pressure several times on the suspicious lesion. Cells are then immediately smeared on glass slides and fixed with alcoholic spray. Signs of dysplasia and malignancy will also be detected cytologically in the upper layers of the squamous epithelium due to the principle of migration of cells from basal to superficial layers. The degree of nuclear abnormality in the surface layers reflects the degree of disturbance of maturation of the whole thickness of the epithelium. Thus, transepithelial sampling is not required to diagnose dysplasia and malignancy of the squamous epithelium on brush biopsies.

4. Assessment of Dysplasia

There are several schemes for grading dysplasia in biopsies of oral precursor lesions. The WHO classification provides a five-step system: hyperplasia, mild, moderate, and severe dysplasia followed by carcinoma in situ [15]. Squamous cell carcinoma will develop from antecedent dysplastic oral mucosal lesions if an early diagnosis has not been made and treatment given. Early diagnosis within stages Tis or T1 correspond to a vastly improved 5-year survival rate when compared with more advanced lesions (96,7%) [17, 18]. It is the task of a cytopathologist to identify nuclear abnormalities

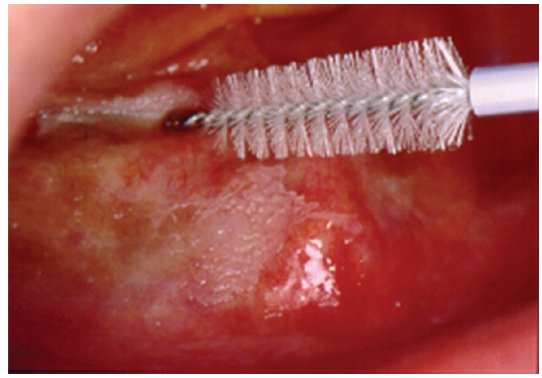


Figure 1: Brush biopsy from an oral verrucous leukoplakia.

in squamous cells collected to predict the histological grade of dysplasia. The diagnostic criteria used are well known and similar to those in cervical exfoliative cytology according to Papanicolaou [4]. Although the degree of dysplasia can be predicted on cytological samples (Figure 2), tissue biopsy is usually performed when dysplasia is detected cytologically, to confirm its grade and exclude the presence of invasion. The latter cannot be reliably assessed by exfoliative cytology alone. However, poor interobserver reproducibility in the histological assessment of oral premalignant lesions is well described [8].

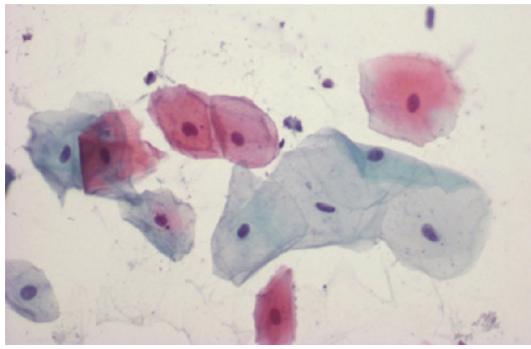
5. Diagnostic Impact

5.1. Spectrum of Cytological Diagnoses. Apart from squamous cell carcinoma and its precursors (dysplasias), further neoplasias can be specifically diagnosed cytologically (e.g., naevuscell naevi, malignant melanomas, basalcell carcinomas, and malignant lymphomas). Moreover, a spectrum of non-neoplastic diseases can be differentiated using exfoliative cytology, for example, pemphigus vulgaris, Candida, herpes simplex, and HPV infections [4].

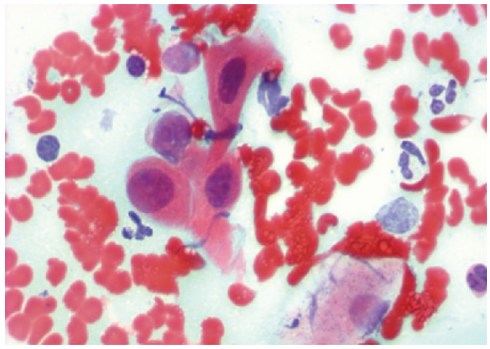
5.2. Diagnostic Accuracy of Cytology. Cytopathologic evaluation of oral brush biopsies from leukoplakias and erythroplakias as a single method yields sensitivities for the detection of oral cancer slightly below those of histopathologic evaluation of scalpel biopsies, reported to be 97,5% [19]. Remmerbach et al. [5, 20] documented 91,3% and 94,6% sensitivity of oral brush biopsy and Maraki et al. [12] even 100% for the detection of oral cancer, including the in situ stage. Respective specificities were 99,5%, 95,1%, and 97,4%. Moreover, 24,1% of cancers were identified in early, curable stages Tis and T1 [20].

It is supported by an increasing number of data that oral cytology is also a valuable technique for the assessment of oral premalignant lesions [3, 12, 21]. Exfoliative cytology has been shown to detect dysplasia in suspicious oral lesions with high sensitivity and specificity by several groups [20].

Up to 5–14% of oral brush biopsies may yield to equivocal cytological diagnoses [5, 20]. Underlying diagnoses are mild, moderate, or marked dysplasia, abnormal regenerating



(a)



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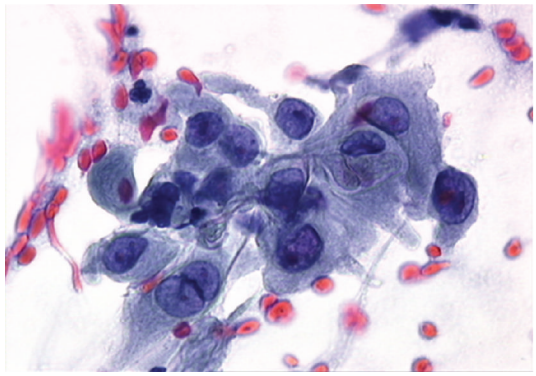

(c)

Figure 2: Normal (a), dysplastic (b), and malignant (c) oral squamous cells from brush biopsy, Papanicolaou stained, 630x.

squamous epithelium, or just scarcity of abnormal cells. In these cases, ancillary methods are desirable that, nevertheless, allow more definite, correct cytological diagnoses.

Meanwhile, use of auxiliary methods such as DNA image cytometry, AgNOR analysis, and multimodal cell analysis has been shown to significantly increase diagnostic accuracy of oral cytology [12, 13, 20, 22, 23]. These methods are only applied on those samples that reveal doubtful or suspicious (dysplastic) cells, on neither cytologically normal nor frankly malignant ones.

6. Auxiliary Cytometry

DNA image cytometry is based on microdensitometric DNA measurements of several hundred atypical cells in routine

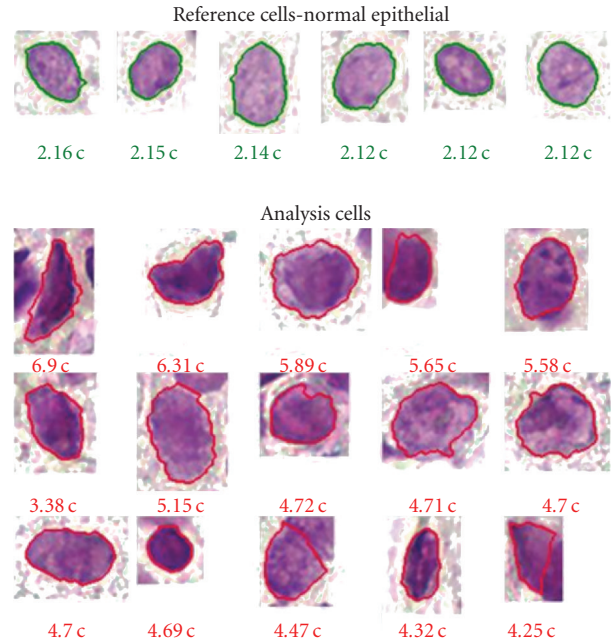

Figure 3: Six nuclei from normal and Feulgen-stained oral squamous cells with regular DNA content (green) as internal reference (around 2,0 c) and 15 from atypical cells with abnormal DNA content (red) between 4.25 c 6.90 c, indicative of malignancy.

cytological specimens (Figure 3). It aims to distinguish true prospectively malignant lesions (dysplasias) from microscopically atypical or otherwise doubtful ones. The biological basis of this ancillary method is chromosomal aneuploidy which is an accepted marker of malignant transformation of cells if it occurs clonally [24]. The cytometric DNA aneuploidy (Figure 4) utilizes the fact that gains or losses of chromosomes or their parts result in a plus or minus of more than 10% of nuclear DNA mass in a growing cell population (stemline aneuploidy) or if extremely high nuclear DNA values >9 c (single-cell aneuploidy) occur [24]. DNA stemlines (modal values) outside 2 c, 4 c, or 8 c ± 10% are regarded as abnormal (resp., aneuploid, Figure 4) [23, 25]. Measurements may be performed on previously stained slides after destaining and Feulgen restaining. Morphologically suspicious cells are interactively selected on a monitor, and internal calibration is performed with normal (e.g., intermediate squamous) cells (Figure 3). The method has been internationally standardized and is applicable to many different epithelial dysplasias [24–26]. After enzymatic cell separation, DNA image cytometry (ICM) can also be applied on formalin-fixed and paraffin-embedded tissues, that is on all histologic routine specimens like biopsies and resected tissues [27]. Thus, even histologic diagnoses of dysplasias can be subjected to DNA cytometry to predict their prospective behavior.

Remmerbach et al. [5] reported a frequency of 13.9% doubtful or suspicious oral cytological diagnoses due to

different grades of squamous dysplasia or abnormal regenerating epithelium. Applying DNA aneuploidy as a marker for prospective malignancy on identical slides, they could improve diagnostic sensitivity of cytology for the detection of oral cancer from 91.3% to 97.8% and specificity from 95.1% to 100%. Thus 29.4% of oral cancers that clinically appeared as leukoplakias or erythroplakias were detected in stages Tis or T1. In a similar study Maraki et al. [12] described a sensitivity of 100% and specificity of 97.4% for the combined cytological and DNA cytometric evaluation of oral leukoplakias and erythroplakias. 8.1% of their cytological diagnoses had been equivocal. DNA-ICM was only applied if one of the above-mentioned diagnoses (mainly dysplasias) had occurred. Seven cases in which combined cytological/DNA cytometric diagnosis of early oral cancer was achieved up to two and half years before definitive biopsy diagnosis have been published [12, 13]. Thus DNA-ICM may help to predict the prospective behavior of cytologically suspicious lesions, as the positive predictive value of DNA aneuploid findings was reported to be 100% and the negative value 98.1% [13, 20].

Another auxiliary method that allows assessment of potential malignancy of dysplastic or regenerating cells is AgNOR analysis. AgNORs represent silver-stainable nucleolar organizer regions (Figure 5). Their number and size are related to protein synthesis. Remmerbach et al. [13, 23] showed that counting the number of silver nitrate-stained nucleolar organizer regions (AgNORs) in about 100 atypical squamous cells allows 100% sensitivity and specificity of oral cancer detection on brush biopsies.

Both methods, DNA-ICM and AgNOR analysis, may even be performed sequentially on identical cells (Figure 5). This type of multimodal cell analysis is especially useful if only few atypical cells are available [23]. Thus, AgNOR analysis can be combined with DNA-ICM if the latter does not yield an unequivocal result.

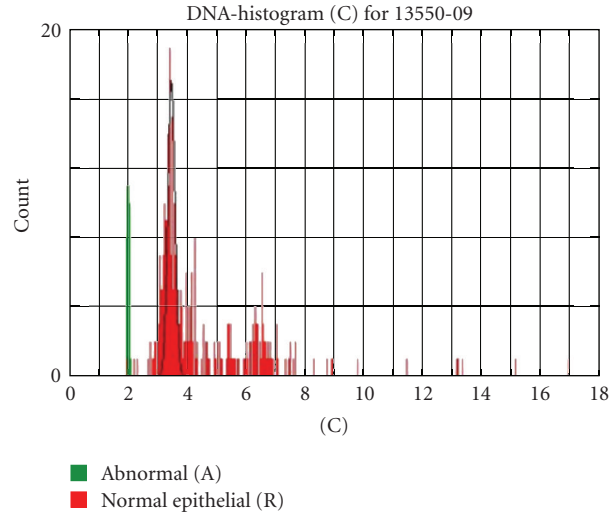

Figure 4: DNA histogram of in-situ oral carcinomas cells, revealing abnormal stemlines (red) at 3.5 c and 6.5 c, and values up to 17 c (DNA aneuploidy), indicative of malignancy. Normal epithelial cells (green bars) at 2 c.

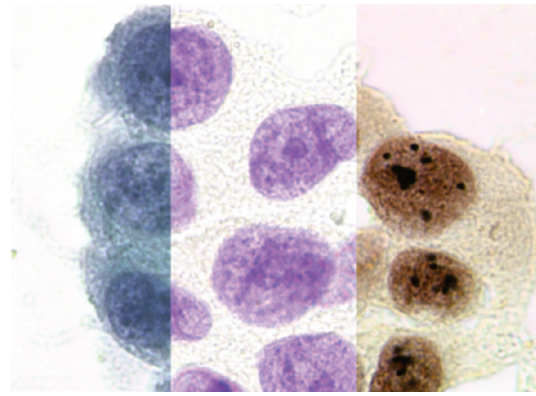

Figure 5: Illustration of three sequential stainings of identical oral cancer cells as performed in multimodal cell analysis [27]: Papanicolaou, Feulgen for DNA analysis, and silver nitrate for AgNOR analysis. Black dots represent AgNORs.

7. Role in Therapy

Treatment method of choice in patients with squamous cell carcinomas of the head and neck area is still surgical resection of the tumor and dissection of the regional lymph nodes. Although options for repair and restoration (e.g., free flaps) of skin and bone defects after primary surgery have improved significantly in the last decades, patients with squamous cell carcinomas of the oral cavity have only a fair prognosis with an overall 5-year survival rate of about 45% [1]. This figure has not substantially improved during the past 30 years [2, 28, 29]. Locoregional failure after surgery or even after combined surgery and radiotherapy is the main cause of death in patients with squamous cell carcinomas of the mandibular region and the maxilla. The main principle in tumor surgery is the effort to achieve tumor-free resection margins.

Several authors have evaluated the relationship between locoregional recurrence of the tumor and the status of the resection margins [30, 31]. The prevalence of tumoral infiltration at the resection margins varies from 3.5% to 60% [30] and is usually an indicator for additional excision, postoperative irradiation, and strict followup [32]. The recurrence rate in patients with positive surgical margins treated only by surgery ranges from 36% [31] to 64% [30], and when postoperative radiotherapy is used, the recurrence rate decreases to 31% [30]. Due to the fact that it can be difficult to distinguish between squamous cell carcinomas and other lesions of the oral mucosa using only haematoxylin and eosin-stained sections [33] the resection margins are routinely examined by immunohistology. Nevertheless, the histological diagnoses of oral mucosa lesions fail sometimes [34, 35]. These days, an alternative method for the examination of oral lesions is exfoliative cytology. It is based on the technique of Papanicolaou, which is accepted worldwide, as a successful method in order to screen for epithelial dysplasias in situ or invasive carcinomas of the uteri cervix. Moreover,

DNA image cytometry has been introduced for diagnosis of malignant transformation of squamous epithelial cells as an adjuvant tool to the cytological examination [20, 36]. This is used to detect the cytometric equivalent of chromosomal or DNA aneuploidy [37], which is accepted as a marker for the neoplastic transformation of cells. DNA image cytometry has been introduced as an adjuvant tool for the detection of these cell transformations in oral mucosa [20, 36]. The detection of DNA aneuploidy has been described as a diagnostic aid for the identification of prospective malignancy in various organs for example in dysplasias of the uterine cervix [38], suspicious cystic lesions of the neck, [39] or bile duct brushings [40]. The positive predictive value of DNA aneuploidy for the subsequent deletion of histologically confirmed cancer was 100% in cells of these tissues. In another study, the additional value of DNA image cytometry regarding the occurrence of a locoregional relapse was assessed [27]. In this study adjuvant use of DNA image cytometry showed a high positive predictive value of 87.5% with respect to the local recurrence of head and neck squamous cell carcinomas. Recently, Brandizzi and coworkers reported a ploidy analysis in oral squamous cell carcinomas using methodologic adjustments to improve the accuracy of the measurements of aggressiveness of prognostic value. Several indices of aggressiveness were analyzed in relation to the clinical-pathologic data and evolution of the patients. Two indices had a prognostic value of the degree of aggressiveness of oral SCC [41].

Taking into account that the diagnosis of tumor infiltration in the resection margins has often serious consequences (followup resection and/or postoperative irradiation), the presence of aneuploid cells could also change the treatment. However, it is unclear if these aneuploid cells cause the locoregional tumor relapse. Unfortunately, up to date no studies exist which confirm this. Thus, it has to be investigated in a consecutive clinical trial, whether the additional or modified treatment leads to a longer relapse-free period.

In conclusion, the additional use of DNA image cytometry is a reasonable tool for the assessment of the resection margins of SCCs. DNA image cytometry could help to find the appropriate treatment option for the patients and thus might improve their prognosis.

8. Followup Care

Local recurrences of oral cancer after operation are frequent events, more often following R 1/2—but even after R0—resections [27]. Exfoliative cytology allows the non-invasive evaluation of macroscopically suspicious mucosal lesions that may appear after resection. As brush biopsies are better tolerated by patients than scalpel biopsies, they may be performed more often. Thus, recurrences may be identified earlier.

9. Conclusion

DNA image cytometry has tremendous impact on early diagnosis and therapeutical considerations in head and neck squamous cell cancer. While oral lesions that macroscopically are urgently suspicious for cancer shall be submitted to scalpel biopsy and histologic evaluation, the majority of facultatively precancerous lesions, such as leuko- and erythroplakias or even persistent lichen planus lesions, may be assessed by brush biopsy and cytology. As this non-invasive procedure is well tolerated by patients, more lesions may be screened and thus more oral cancers may be found in early, curable stages. Oral brush biopsies can easily be performed by dentists, dental surgeons, and general practitioners. While sensitivity of exfoliative cytology alone is about 4% less than bioptic histology, the combination of the latter with DNA image cytometry reaches the same diagnostic accuracy as the former. As clonal chromosomal aneuploidy and DNA aneuploidy mostly precede cytological and histological evidence of malignancy in the squamous epithelium, its detection allows the diagnosis of oral squamous cell carcinomas up to two years earlier. Moreover, the additional use of DNA image cytometry is a reasonable tool for the assessment of the resection margins of squamous cell carcinomas. DNA image cytometry could help to find the appropriate treatment option for the patients and thus might improve their prognosis.

Finally, diagnostic DNA image cytometry is an accurate method and has internationally been standardized. Actually, it is paid by the German health insurances.

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Review Article

Salvianolic Acid B, a Potential Chemopreventive Agent, for Head and Neck Squamous Cell Cancer

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Head and neck squamous cell cancer (HNSCC) is one of the top ten cancers in the United States. The survival rate of HNSCC has only marginally improved over the last two decades. In addition, African-American men bear a disproportionate burden of this preventable disease. Therefore, a critical challenge of preventive health approaches is warranted. Salvianolic acid B (Sal-B) isolated from Salvia miltiorrhiza Bge, which is a well-know Chinese medicines has been safely used to treat and prevent aging diseases for thousand of years. Recently, the anticancer properties of Sal-B have received more attention. Sal-B significantly inhibits or delays the growth of HNSCC in both cultured HNSCC cells and HNSCC xenograft animal models. The following anticancer mechanisms have been proposed: the inhibition of COX-2/PGE-2 pathway, the promotion of apoptosis, and the modulation of angiogenesis. In conclusion, Sal-B is a potential HNSCC chemopreventive agent working through antioxidation and anti-inflammation mechanisms.

1. HNSCC and Chemoprevention

Over 90% of head and neck cancers are squamous cell carcinomas (HNSCC). Oral cancer accounts for a major proportion of HNSCC, which is the sixth most common cancer worldwide. In the United States, oral and pharyngeal cancers alone are diagnosed in about 36,540 Americans annually, and 7,880 are projected to die from these diseases in 2010 according to the American Cancer Society. HNSCC has been less studied compared to other cancers and the incidence of this cancer has not shown any improvement in the last 20 years (Figure 1). The 5-year survival rate for oral and pharyngeal cancers in Caucasian patients is 56%, while for African American men; it is only 34% [1]. In over 50% of first diagnosed cases of HNSCC in African American men, the cancer has already metastasized to other organs, such as the lungs. HNSCC prevention, earlier detection, and viable treatment options are of paramount importance to reduce the cancer incidence, improve patient outcomes and diminish the disparity.

HNSCC have been considered to be a typical multistep carcinogenesis with stepwise accumulations of genetic alterations resulting in aberrant cellular appearance, deregulated cell growth and carcinoma [2]. Patients with early stages of disease still have high risk to develop a second malignancy. A normal epithelial cell can take many years to undergo the multiple cellular and genetic alterations that lead to malignant changes. Thus, it remains an appealing strategy to develop effective, nontoxic and affordable novel pharmacological agents for preventing development of HNSCC and second primary HNSCC [2–5]. Chemoprevention has been considered a rational and appealing strategy to prevent or delay the development of HNSCC, additionally; dietary nutrients such as green tea, β-carotene and vitamin E have been also used as preventive agents [5–8]. Extensive studies have suggested that green tea, one of the most commonly consumed beverages worldwide, can reduce the risk of HNSCC development by inducing antioxidative activity via apoptosis and inhibiting epidermal growth factor receptor related signaling pathway [7, 9, 10]. There have been an increased number of case reports that high doses of green tea beverages cause hepatotoxicity [11]. Both vitamin E and β-carotene revealed the growth-inhibitory effect against lung cancer in cell culture and rodent models. But the promising activities have not translated into clinical success. Indeed, these supplements may actually

lead to unexpected detrimental effects in humans as well as beneficial effects [12, 13]. Hence, the crux is to find an effective, nontoxic and affordable novel pharmacological agent in clinical trials for preventing carcinogenesis and the development of HNSCC as well as second primary HNSCC.

2. Salvianolic Acid B

Radix Salviae miltiorrhizae (danshen or tanshen), the dried root of Salvia miltiorrhiza Bge is very important and popular in traditional Chinese medicine. It that has been widely and successfully used treating and preventing aging diseases, such as cardiovascular and cerebrovascular diseases, and cancers for thousand years and is ranked as a "Super grade" drug recorded in Shen-Nung's Pen-Ts'ao [14]. Currently, danshen has been accepted and used in Japan, the United States and some European countries [14–16]. In the last 50 years, Danshen received more attention by modern scientists that more than 70 compounds, including the hydrophilic depsides derivatives and the lipophilic diterpenoids, have been isolated from the Danshen herb [16, 17]. Salvianolic acid B (Sal-B) is the most abundant and bioactive member of the hydrophilic components in Danshen. Therefore, Sal-B is used as a quality control ingredient and active marker for S. miltiorrhiza Bge products by the National Pharmacopoeia Council of China. Sal-B contains seven phenolic hydroxyls which have been found to be closely related to redox potentials and/or antioxidant activities [18]. The structure of Sal-B is depicted in Figure 2. Sal-B has been studied for its preventive effects against cancer as well as cardiovascular, neurodegenerative, and other diseases [19–23]. The mechanisms mainly contribute to its antiinflammatory and antioxidative properties, modulation of apoptosis, inhibition of platelet aggregation, improved coronary microcirculation, as well as, regulation of angiogenic processes [14, 24, 25]. We will introduce the function and biological activities of Sal-B, validate its efficacy on HNSCC, and discuss the foreground of this component.

3. Antiinflammatory Activities

It appears that there is a general concept that chronic inflammation characterized by continued active inflammation responses and tissue destruction, can be a major cause of cancers and occur during the aging process [26–28]. Mounting studies have reported that Sal-B is capable of preventing the development of cancer; and the possible antiinflammatory mechanisms of Sal-B involve modulating cytokines, Cyclooxygenase-2/prostaglandin E2 (COX-2/PGE-2) pathway [24, 25, 29], NF-κB [30–32], TNF-α [33–35] and MMPs [36–38]. Numbers of clinical experiences indicate its effectiveness and safety in contrast to the disadvantages of nonsteroidal antiinflammatory drugs (NSAIDs). NSAIDs, used primarily to treat inflammation, are associated with several serious side effects including gastrointestinal discomfort, cardiovascular disease and kidney failure [39–42].

3.1. COX-2/PGE-2. COX-2, an inducible form of cyclooxygenase is undetectable in most normal tissues, but abundant in the pathogenesis of inflammatory and neoplastic diseases. Additionally, its principal metabolite PGE2 has pleiotropic effects such as promoting cell proliferation, inhibiting cell death, promoting tumor angiogenesis, and decreasing immunosurveillance. Sal-B has been reported to attenuate significantly COX-2 expression and PGE2 production with or without lipopolysaccharide (LPS)-induced both in vitro and in vivo [24, 25, 29], and which may be attributed to the downregulation of JNK and ERK phosphorylation and blockage of MAPKs phosphorylation [29].

3.2. NF-κB. NF-κB, a protein complex that controls the transcription of DNA, regulates cellular responses as a "first responder" to harmful cellular stimuli, and its aberrant expression is linked to cancer and inflammation [43]. Several experiments clarified that the antiinflammatory effects of Sal-B depend on the inhibition of the NF-κB signaling pathway [30–32]. Moreover, Sal-B attenuates the expression of VCAM-1 and ICAM-1 in TNF-α stimulated human aortic endothelial cell by partial blockage of NF-κB expression [34].

3.3. TNF-α. Tumor necrosis factor-alpha (TNF-α), a representative proinflammatory cytokine damages cell structure and increases endothelial permeability and is involved in systemic inflammation [44]. Sal-B has showed to significantly reduced the production of TNF-α induced by LPS treatment in rat primary microglia in a dose-dependent manner [32]. In addition, Sal-B protects endothelial cell against TNF-α disruption by inhibiting vascular endothelial growth factor (VEGF) and extracellular signal-regulated kinase (ERK) activation [45].

3.4. MMPs. Matrix metalloproteinases (MMPs), inflammatory mediators are expressed in vascular cells in the course of atherosclerosis [46]. Sal-B treatment effectively inhibits MMP-2 and MMP-9 activitation and expression both cell culture and animal models, and it also, downregulates JNK and ERK phosphorylation [36, 37]. Some researchers found Sal-B could suppress high glucose-induced mesangial cells proliferation and extracellular matrix production in a dose-dependent manner, partially through modulating the cell-cycle progress and MMP-2 and MMP-9 activities [30, 31].

4. Antioxidative Activities

A vast amount of evidence suggests that overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can damage cellular lipids, inhibit the normal function of proteins or DNA, and are associated with the pathogenesis of atherosclerosis, cardiovascular diseases, hypertension, ischemia/reperfusion injury, neurodegenerative diseases, and cancer [47, 48]. Sal-B, as an antioxidant neutralizes direct ROS attacks and terminates free radical-mediated oxidative reactions to protect the human body from such diseases [49].

4.1. Radical Scavenging. Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radicals and hydrogen peroxide are chemically reactive molecules derived from oxygen. They usually contain one or more unpaired electrons in the atomic or molecular orbital [50]; therefore, they can easily participate in redox reactions and they play a crucial role in biological systems [51, 52]. Excess ROS can disturb the equilibrium status of prooxidant/antioxidant reactions, leading to the disruption of cellular functions in contrast to low/moderate concentrations that occur in response to induction of a mitogenic reaction and normal function of several cellular signaling pathways [53, 54]. There is increasing evidence that Sal-B has the capability to scavenging free radicals including superoxide anion, hydroxyl, DPPH (1,1-diphenyl-2-picryl-hydrazyl) and ABTS (2-azino-bis(3-ethylbenzthiazoline-6- sulfonic acid)) radicals in addition to hydrogen peroxide due to redox properties of the phenolic structure [55]. Sal-B showed a high antioxidant capacity in terms of neutralizing free radicals, as well as exhibiting significantly higher scavenging activity than l-Ascorbic acid (vitamin C) [56, 57]. 50% radical scavenging activity at a concentration of the Sal-B lower ~50% and ~40% than Vitamin C in DPPH and ABTS assays, respectively (1.81 ± 0.01 versus $3.44 \pm 0.03\,\mu$g/ml, 1.43 ± 0.01 versus $2.50 \pm 0.02\,\mu$g/ml) [57].

4.2. Antioxidant Activities. Free radicals have been elucidated to cause oxidative damage to cellular components, including attacks on DNA, oxidation of proteins and production of lipid peroxidation, these processes lead to disorder in cellular, tissue and organ function [52, 58, 59]. Sal-B has been reported to be a powerful and effective antioxidant, not only reducing lipid peroxidation, but also rescuing the loss of antioxidant enzyme activities against fibrosis and ischemia-reperfusion injuries [60, 61]. Some studies have reported that Sal-B can protect the brain and heart from ischemia-reperfusion injury by improving the recovery of motor function via regulating energy metabolism and maintaining the balance of free radicals such as SOD, GSH, and ATP levels against lipid peroxidation and superoxide anion production [62–65]. In the hepatic stellate cells (HSCs), Sal-B exerts suppressive effects on ROS to inhibit the proliferation and lipid peroxidation of HSCs through inhibiting NADPH oxidase and TGF-β1 secretion [49, 66, 67]. Studies have shown that ROS leads to the oxidation of low-density lipoproteins and accumulates within plaques and contributes to the atherosclerosis [66, 68]. Sal-B was identified to be a potent antioxidant, endothelial-protecting agent, an inhibitor that suppresses the expression of ICAM and VCAM, capable of inhibiting LDL oxidation and also inhibits ox-LDL induced endothelial injuries [69]. In sum, the antioxidative properties of Sal-B are closely associated with its protective effect of aging diseases [70].

5. HNSCC and Sal-B

Cancers have a close and delicate relationship with inflammation [26, 71, 72]. Inflammation often exists in the tumor microenvironment and is induced by inflammatory mediators produced by the tumor [73–75]. HNSCCs are highly inflammatory and aggressive, and they overexpress a number of inflammatory mediators such as COX-2, EGFR, VEGF and MMPs [76–78]. Sal-B, as chemopreventive agent exerts its effects by inhibiting tumor initiation and development; its anticarcinogenic activities have been clearly demonstrated in both cell cultures and animal models [24]. Furthermore, research has also shown that the combining Sal-B with other preventive agents is more effective than single-agent chemoprevention [25].

We tested the anticancer function of Sal-B in five human HNSCC cell lines (JHU-6, JHU-11, JHU-13, JHU-22 and JHU-29) that Sal-B significantly inhibit the cell growth in cultured cells [24]. In the animal experiments, HNSCC solid tumor volume in Sal-B treated group were significantly lower than those in untreated control groups [24]. The outcome is consistently obtained in human breast and prostate cancer cell lines. We found that Sal-B selectively suppresses COX-2-related mRNA and protein expression instead of housekeeping enzyme of COX-1 in the presence or absence of LPS stimulation. It seems that the chemopreventive effects of Sal-B depend on COX-2 expression levels, the higher the expression of COX-2 the more sensitive is Sal-B HNSCC cell growth-inhibition and PGE2 reduction. In addition, Sal-B induced caspase-dependent apoptosis by cleavage of a caspase substrate, poly (ADP) ribose polymerase (PARP). Sal-B also decreased the cellular amounts of antiapoptotic proteins such as NF-κB, MDM-2, Bcl-2 and Bcl-xL and increased proapototic proteins such as p53 and caspase 3 [24, 79]. The mechanism of cancer-prevention was attributed to the COX-2/PGE2 inhibition and apoptotic pathway induction. PGE2, one of important prostaglandin product of COX-2 is involved in chronic inflammation [80].

A promising strategy to enhance the cancer-preventive efficacy is to use two anticancer agents in combination, which may produce synergy and lower the dose required for each agent [79, 81]. Celecoxib, a selective COX-2 inhibitor has been reported to have cancer-preventive effects in various types of cancers including HNSCC [82]. However, it was found to be associated with a dose-dependent cardiovascular morbidity that limited its long-term use. Hence, we decided to use Sal-B combined with low-dose celecoxib in order to increase the anticancer efficacy and reduce drug side effects. The outcome showed that the combination of half-dose of Sal-B and celecoxib greatly enhanced the inhibition of HNSCC cell proliferation compared with either Sal-B or celecoxib alone both in cell culture (JHU-06, JHU-011, JHU-013 and JHU-022) and tumor xenografts. The combination was associated with profound inhibition of the COX-2/PGE2/EGFR pathways, enhanced induction of apoptosis, and reduced the side effects of celecoxib due to dose reduction at the same time [25].

The anticancer effects of Sal-B were also found in 7,12-dimethylbenz-[a]anthracene-(DMBA) induced oral carcinogenesis in hamsters [83]. Experiments showed that Sal-B treatment significantly decreased the oral cancer incidence. Antiangiogenesis may be one of the possible mechanisms of inhibiting malignant transformation of oral precancerous

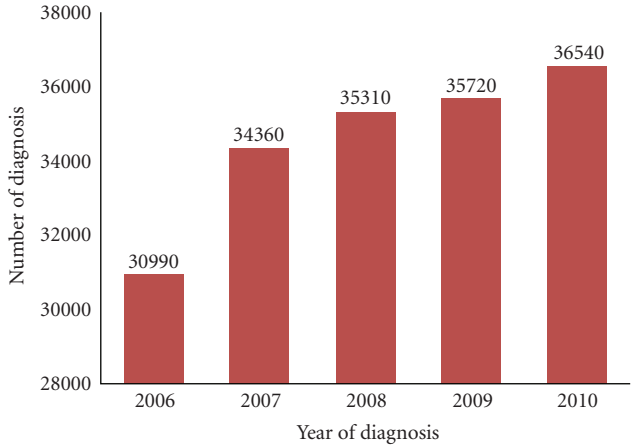

Figure 1: Incidence of oral cavity and pharynx cancer.

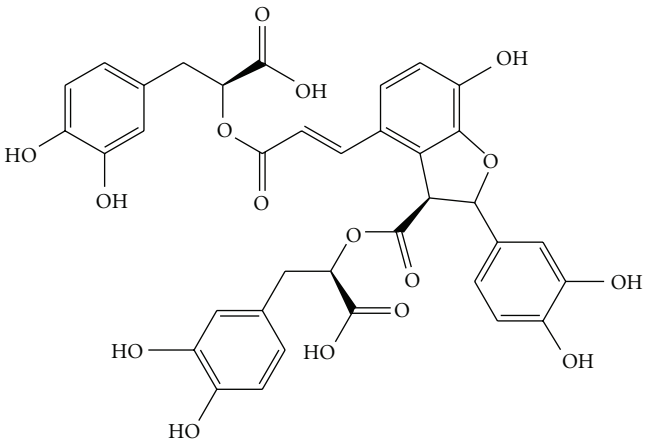

Figure 2: Chemical structure of Salvianolic acid B.

[75, 84]. The formation of microvessels, and the expression of proangiogenic factors HIF1α and VEGF, was inhibited by Sal-B.

Recently, we were inclined to accept the concept that the prevention is better than a cure. Actually, prevention is more valuable to reduce the incidence of HNSCC than increase survival rate [85, 86]. Anticancer properties of Sal-B are able to prevent and delay the malignant conversion of premalignant lesions and/or cell growth via inhibition of inflammation and angiogenesis and reduction of apoptosis (Figure 3).

6. Problem and Future Prospects

Sal-B as a popular compound of Traditional Chinese Medicine has been studied for its preventive effects against HNSCC. Most of the proposed beneficial effects have been attributed to antioxidative and antiinflammatory effects. It is known that the relationships among oxidation, inflammation and cancer are considered to be extremely complex. Firstly, chronic inflammation increase the risk of developing many types of cancer including HNSCC, and inflammatory

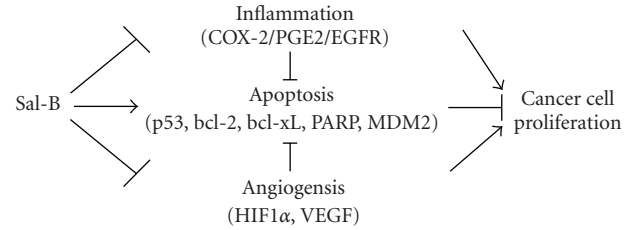

Figure 3: Possible anticancer activites of Salvianolic acid B.

cells, chemokines and cytokines are present in the microenvironment of all tumors [71]. Secondly, ROS, an endogenous class of carcinogens trigger the mutation of the cells have been demonstrated in the principal step of carcinogenesis and contribute to cancer progression. Moreover, cancer cells frequently produce more ROS than normal cells [87, 88]. Thirdly, activated inflammatory cells generate ROS and reactive RNS in response to proinflammaory stimuli, which can function as chemical effectors in inflammation-driven carcinogenesis; ROS-induced oxidations are implicated in inflammation via regulation signal pathways and related enzymes such as COX-2 [88, 89]. Sal-B, a natural anticarcinogenic agent with antiantioxidant and antiinflammatory activities, reacts easily with free radicals and inhibits effectively COX-2/PGE-2 pathway as well as regulating related cell signal pathways. It is difficult to distinguish which is determines prevention to HNSCC because oxidation, inflammation and cancer are intertwined in a complex web. Sal-B has been showed a significant advantage for the prevention and treatment in HNSCC due to effectiveness and nontoxic nature.

The discovery of Sal-B anticancer properties is followed a path of from "bed" to "bench". A thousand years clinical practices demonstrate that Danshen is able to effectively and safely prevent and treat aging diseases, such as cardiovascular diseases and cancers. The mechanism studies of Danshen have been concerned until latest 50 years. Sal-B is a most abundant and bioactive member of hydrophilic components in Danshen. The same as Danshen, Sal-B is a safer agent with no major side effects [20]. Due to our aging society, both cancer and cardiovascular diseases have increasingly become the two major killers and human health hazards. In recent years, the amount of cytotoxic agents and targeted therapies used to treat HNSCC, include classic chemotherapeutic agents, chemoprevention agents such as COX-2 inhibitors, monoclonal antibodies targeting tyrosine kinase receptors, small molecule tyrosine kinase inhibitors, and antiangiogenic drugs [90]. Unfortunately, these result in cardiovascular toxicity. Especially, celecoxib, a selective COX-2 inhibitor has been required restricted use owing to its potential to effects the cardiovascular system in long-term use by the Food and Drug Administration and was announced the early cessation of a cancer-prevention clinical trial. It is hailed that Sal-B is a not only cancer-preventive agent but an also cardiovascular protective agent. Sal-B inhibits cancer cell proliferation *in vitro* and *in vivo* in addition to regulating the microenvironment. In a study with

celecoxib, Sal-B resulted in platelet aggregation, playing a key role in cardiovascular protection [20]. Since Sal-B exerts dual pharmacy characteristics, it will lead to broad applications in the future. But related-mechanisms are still not fully understood, we need specific and profound studies about Sal-B.

In addition, some studies also reported Sal-B was released from nanotechnology samples faster and had increased antioxidant activity compared to the traditionally-powdered samples [89, 91, 92]. Combined with chemopreventive agents of traditional Chinese Medicine will further promote development of Sal-B.

We found an interesting and confusing phenomenon that Sal-B showed a distinct attack and protective features in different cell lines. Some studies reported Sal-B protected the SH-SY5Y neuroblastoma cells, hepatocyte and bone marrow stem cells against apoptosis by relieving oxidative stress and modulating the apoptotic process [93–95]; on the other hand, Sal-B has been revealed to activate apoptosis pathways in order to inhibit cancer cell proliferation [24, 25]. It seems to be controversial; whereas, the mechanism of signal regulation is very complex and therefore the role of Sal-B depends on the type of cell line, the microenvironment. Different concentrations of ROS showed both the induction and inhibition in cancer development. To define an accurate mechanism of Sal-B, particularly which are Sal-B exact targets, still calls for more studies and developments.

In conclusion, Sal-B, a natural antiinflammatory (selective COX-2 inhibitor) and antioxidative agent has chemopreventive activity on HNSCC; due to its effectiveness and safety it could have much more commercial value for food and medicine purposes.

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Review Article

Contact Endoscopy as a Novel Technique in the Detection and Diagnosis of Mucosal Lesions in the Head and Neck: A Brief Review

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Background. There are a variety of described noninvasive optical detection techniques for evaluation of head and neck mucosal lesions. Contact endoscopy is a promising method of *in vivo* microscopic examination whereby a rigid telescope is placed on a previously dye-stained mucosa allowing evaluation of the superficial cell layers of the epithelium. This technique produces real-time, magnified images of cellular architecture of surface mucosa comparable to histology without the need for biopsy. In this review, we will briefly summarize the efficacy of CE in the detection of precancerous and cancerous mucosal lesions and its potential as a novel technique in early diagnosis, monitoring, and preoperative assessment of mucosal lesions of the head and neck. *Methods*. PUBMED, MEDLINE, and COCHRANE search revealed five prospective articles on contact endoscopy for the diagnosis of mucosal lesions in the head and neck. *Results*. The literature search yielded five prospective studies examining contact endoscopy for the diagnosis of benign versus malignant head and neck mucosal lesions. These reported a sensitivity and specificity of 77–100%, specificity of 66–100% and an accuracy of 72–92%. *Conclusion*. Contact endoscopy is a promising optical technology that may be a useful adjunct in the evaluation and diagnosis of benign and malignant head and neck mucosal lesions. Future prospective randomized double-blind studies of this detection method are required.

1. Introduction

The vast majority of cancers in the oral cavity and in the head and neck are squamous cell carcinomas (SCCs). It is the sixth most common cancer worldwide, and its incidence is rising in industrialized nations [1, 2]. Head and neck cancer is a major cause of morbidity and mortality. Many cancers of the head and neck arise from precancerous lesions such as leukoplakia. Some studies quote leukoplakia as having a 10% chance of transformation into carcinoma. Similarly, other benign lesions of the oral cavity such as lichen planus may have a prevalence of 0.5–2% in the general population and may have a risk of malignant transformation of 1% [3]. Thus, early detection and diagnosis of suspicious mucosal lesions is essential.

Many benign oral mucosal lesions are not cancerous which presents a clinical dilemma to the physician. Furthermore, precancerous lesions such as leukoplakia may exhibit mild structural alterations in the mucosa that can be difficult to distinguish from normal healthy tissue. Currently, obtaining histopathology via biopsy is the gold standard of diagnosis; however, this procedure can pose significant morbidity to the patient such as the risk of bleeding, wound infection, and potentially impairment of speech and swallowing if multiple biopsies are performed.

Moreover, it becomes a clinical challenge to monitor patients for progression of diffuse dysplasia or leukoplakia, and many of them may require multiple biopsies over many years. The discomfort of biopsy and compromisation of tissue integrity can lead to problems with future biopsy interpretation or in the case of laryngeal biopsy, considerable problems in individuals with high vocal demands [4]. Subsequently, any technique that can yield histopathological information without injuring tissue has obvious advantages over biopsy.

Detailed examinations of the texture, color, contour, and extent of mucosal lesions have been performed utilizing many instruments such as the Hopkins' rod-lens scopes, flexible endoscopes, direct laryngoscopes, and advances in microlaryngoscopic visualization techniques. However, these methods are limited by their inability to provide histopathological data during the clinical examination.

As a result, over the last decade, technological advances in optical imaging detection techniques have emerged with a variety of methods employed to facilitate detailed examination and provision of histopathological information of mucosal lesions. Examples of such novel optical techniques include: aminolevulinic acid-induced fluorecence, autofluorescence, confocal endomicroscopy, and contact endoscopy.

Aminolevulinic acid-induced fluorescence is a technique whereby neoplastic cells undergo preferential fluorescence after aminolevulinic acid (ALA) has been applied to the mucosa surface. In the presence of ALA, tumors have selective accumulation of protoporphyrin which can be differentiated from healthy tissue. Once this "dye-like" substance has been applied, mucosa containing neoplastic cells will fluoresce orange red and normal mucosa will retain the normal green fluorescence. Coupled with autofluorescence, several authors have noted that these techniques can diagnose laryngeal carcinoma and dysplasia with good accuracy [5].

Autofluorescence was first described in identification of neoplastic cells of the larynx by Harris et al. [6]. Tissue fluorescence is induced by short-wave blue light of the visible spectrum. Certain molecules then transform into photonic energy, which is emitted as long-wave scattered light which can be detected. Each molecule has a characteristic fluorescence spectrum dependent on the excitation light. These fluorescent molecules are called fluorophores. The autofluorescence imaging method detects the fluorecence given off by the different concentrations of fluorophores seen in normal and neoplastic mucosa. Normal healthy mucosa fluoresces bright green while neoplastic mucosa appears red violet [7]. Thus, autofluorescence videoendoscopy for photodiagnosis of head and neck squamous cell carcinomas has been described as being quite accurate with good sensitivity and specificity in several studies [6–16].

Unlike ALA and autofluorescence where histological detail is not appreciated, other optical techniques such as narrow-band imaging endoscopy (NBIE) allows increased visualization of histological detail. NBIE uses filtered light with wavelengths preferentially corresponding to peaks of absorption of hemoglobin to enhance superficial neoplasms based on their neoangiogenic pattern. These light wavelengths penetrate superficial mucosal and deep submucosal layers to enhance capillary and submucosal vasculature. The obtained image is further enhanced by using high-definition television (HDTV) [17]. Carcinomas can then be identified based on the changes in the microvascular pattern of the mucosal lesion. Several studies have shown good sensitivity, specificity, negative and positive predictive value, and accuracy in detection of squamous cell carcinoma of the oral cavity, oropharynx, larynx, and esophagus [17–20].

However, instead of solely relying on neoangiogenic patterns for diagnosis of carcinoma, further histological detail can be obtained with the use of confocal endoscopy which is an *in vivo* optical imaging method whereby mucosal lesions can undergo significant magnification to allow examination of cellular histology. This technique also allows reconstruction of three-dimensional structures based on the acquired images. Utilization of various stains to help highlight cellular structures has been tried by some authors to distinguish normal from invasive carcinoma cells. The utility of this new technology is highlighted in its capability to distinguish between benign or low-grade mucosal dysplasia thereby potentially reducing unnecessary biopsies [21].

Contact endoscopy is another novel noninvasive optical diagnostic imaging method that allows *in vivo* and in situ examination of the cellular architecture of the superficial layers of the mucosal epithelium. Magnified images are obtained using Hopkins' rod-lens endoscope placed on the surface of the dye stained mucosal tissue. This technique allows assessment of precancerous and cancerous lesions *in vivo* and has significant potential in the histopathologic diagnosis of many suspicious head and neck mucosal lesions without tissue biopsy.

CE was originally described and used by Hamou in 1979 as a technique for visualization of cervical and uterine epithelial cells for screening and diagnosis of cervical and uterine pathology [22]. The first reported use of CE in otolaryngology head and neck surgery was by Andrea et al. as a diagnostic tool in the evaluation of various pathologies in the larynx in the 1990s [4, 23–30]. They were able to visualize and diagnose laryngeal mucosal pathology from the magnification of vocal fold epithelium and microvasculature during microlaryngoscopy after staining the vocal cords with methylene blue dye.

Current contact microlaryngoscopes come in a variety of lengths, diameters and viewing angles. Diameters, of these scopes come as either 4 mm or 5.5 mm and lengths of 23 cm and 18 cm. Straight forward (O°) and Forward-Oblique telescopes (30°) are also available, and all are capable of 1x, 60x, and 150x magnification. These endoscopes require a high intensity xenon light source, and images can be digitally captured for real-time photographic and video documentation, Figures 1 and 2.

The most basic technique of CE involves staining of the superficial cells of the mucosa with a contrast dye, 1% methylene blue (MB) after which the magnifying endoscope (Karl Storz 8715 AA, Tuttlingen, Germany) 0° is then placed in contact against the mucosal surface, and the documented magnified cytological images (at 60x or 150x)

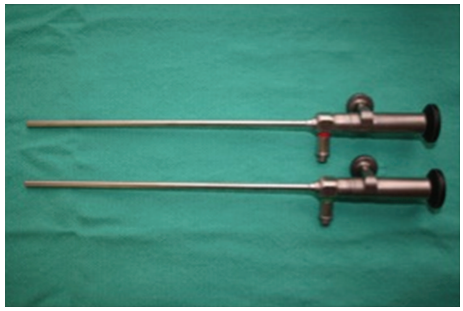


Figure 1: Top (zero-degree) and bottom (thirty-degree) contact endoscopes.

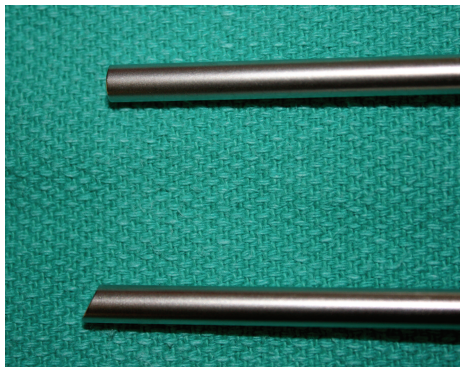


Figure 2: Closeup of endoscope tips. Top (zero-degree) and bottom (thirty-degree) contact endoscopes.

are then recorded, Figure 3. Both a cytopathologist and an otolaryngologist can then assess these images, comparable to histology, Figure 4. Contact endoscopy and its efficacy in head and neck oncology, advantages, limitations, and future potential diagnostic utility will be briefly reviewed in this article.

2. Methods

The literature search was conducted using the following key terms: "contact endoscopy", "contact microlaryngoscopy", "Aminolevulinic acid induced fluorecence", "autofluorescence", "confocal endomicroscopy", "oral mucosa", "oral cavity", "larynx", "oropharynx", "hypopharynx", "head and neck carcinoma", "leukoplakia", and "lichen planus." Significant publications were identified using MEDLINE, COCHRANE and PUBMED databases. Relevant search terms and combinations using Boolean operators were performed, and relevant article selection was limited to the prospective, human and English studies without restriction to year of publication. All appropriate article references were searched and cross-referenced.

3. Results

Five prospective articles were examined. Efficacy data from these studies are summarized in Table 1.

Figure 3: An otolaryngologist performing contact endoscopy of an oral mucosal lesion.

Warnecke et al. [30] examined 42 consecutive patients at a tertiary care center with suspicious lesions of larynx, pharynx, and esophagus under general anesthesia. Indication for endoscopy was a tentative clinical diagnosis of malignant tumor of oropharynx. All were biopsied postendoscopy. The results obtained by the cytopathologist and otolaryngologist were based on images generated from the CE. The histopathology obtained was considered the gold standard. All of the samples obtained were blinded. They found that the more experienced the examiner, the higher the sensitivity of CE was in the diagnostic differentiation of benign versus malignant mucosal lesions.

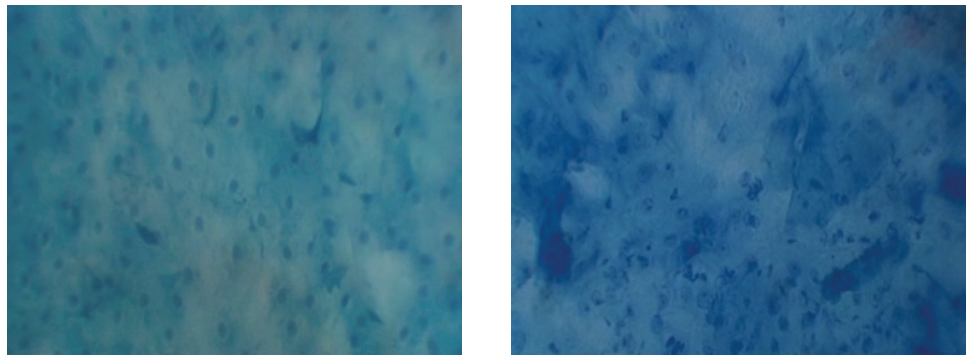
Cikojević et al. [4] examined the utility of CE in intraoperative diagnosis of laryngeal pathology. They included 142 patients undergoing microlaryngoscopy at their institution with various laryngeal diseases all underwent CE and subsequent biopsy for histopathological diagnosis. All malignant lesions identified by CE was confirmed by histopathology, but CE did not identify malignancy in 10 patients diagnosed histopathologically thus giving CE a sensitivity of 79.6%, specificity of 100%, and accuracy of 93%.

Tarnawski et al. [31] examined 54 patients with various laryngeal pathology intraoperatively during microlaryngoscopy. CE was performed, and biopsies were taken from all patients for histopathological diagnosis. Their results were based on computer-assisted analysis of all CE images based certain nuclear morphometric parameters to determine benign from malignant lesions. Thus, based on their computer-assisted analysis of CE images, their sensitivity was 91% and specificity 81%.

Pak et al. [32] prospectively examined 64 patients with previous irradiation for nasopharyngeal carcinoma (NPC). All patients were examined with contact rhinoscopes under local anesthesia and biopsy of the area under examination was done . In all 5 cases of malignancy, CE and histological diagnosis directly correlated with each other.

Most significantly, for the prediction of persistent and recurrent disease, sensitivity and specificity for CE was 100% with an accuracy of 92.1%.

Finally, Arens et al. [26] pilot study examined 83 patients using both autofluorescence and contact endoscopy during microlaryngoscopy. For contact endoscopy, the calculated



(a) (b)

Figure 4: Images (150x magnification) of a benign (normal mucosa on pathology) and malignant (squamous cell carcinoma on pathology) oral cavity lesion demonstrating magnified cellular architecture as acquired by contact endoscopy.

Table 1: Summary of efficacy data from prospective contact endoscopy trials.

Author	Study type	Number of patients	Number of males (M) and females (F)	Average age (age range)	Type of institution	Head and neck subsites	Type of lesions examined	Sensitivity %	Specificity %	Accuracy %
Warnecke et al. [30]	Prospective	42	M = 30 F = 12	55.6 (21–76)	Tertiary	Pharynx, hypopharynx, larynx	Normal and inflamed mucosa, dysplasia, SCC	90	93.8	88
Cikojević et al. [4]	Prospective	142	M = 101 F = 41	N/A (19–81)	Tertiary	Larynx	Benign, hyperplasia, dysplasia (grades I, II, III), papilloma, CIS, SCC	79.6	100	93
Tarnawski et al. [31]	Prospective	54	M = 22 F = 17	51.9 (47–69)	Tertiary	Larynx	Normal mucosa, mild & severe dysplasia, SCC	91	81	N/A
Pak et al. [32]	Prospective	64	M = 54 F = 10	42 (21–77)	Tertiary	Nasopharynx	Metaplasi, atypia, granulation tissue, carcinoma	100	100	92.1
Arens et al. [26]	Prospective	83	N/A	N/A	Tertriary	Larynx	Normal mucosa, dysplasia (grades I, II, III).	94.7	95.5	94

N/A=not available; CIS=carcinoma in situ; SCC=squamous cell carcinoma.

sensitivity was 94.7, specificity of 95.5 and an accuracy of 94%.

In summary, authors of the above prospective trials have obtained the following results: a sensitivity of 79–100%, a specificity of 81–100%, and an accuracy of 88–94%. Overall, it appears that sensitivity, specificity, and accuracy of CE are similar across the trials.

4. Discussion

Since the development of contact endoscopy, this technology has been used successfully by several authors in analyzing and diagnosing various pathologies of the larynx, oral cavity, oropharynx, and nasopharynx via "real-time" examination of mucosal cytological detail [4, 26–31, 33–36]. Despite its introduction into otolaryngology, CE has yet to find a place in routine clinical practice despite its potential advantages.

From the above clinical trials, CE appears to have good sensitivity, specificity, and accuracy as a noninvasive method for distinguishing between benign and malignant mucosal lesions in the head and neck. However, some authors state that it may be difficult for CE to detect mild (grade I) mucosal dysplasia because most of the cellular anomalies occur in at the level of the basal epithelium, and this technique can only examine cellular architecture found at the superficial epithelial layers [4, 21].

Despite this limitation, other authors have found CE diagnosis to correlate well with histopathological findings. Most significantly, CE accurate ability to diagnose and tease out the histological differences between squamous

metaplasia, atypia, and carcinoma even in the presence of irradiated mucosa was highlighted by the study performed by Pak et al. [32]. At present, most authors seem to agree that it has significant potential as a noninvasive detection method that could play a role as a future substitute for histological examination.

There are several advantages of contact endoscopy. Most significantly, it offers a noninvasive, rapid, and repeatable *in vivo* assessment of the cytological architecture while avoiding the need for an invasive biopsy and its associated risks. CE provides immediate results, with the possibility of examining multiple mucosal areas in a short time. CE can also assess a wider surface mucosal area, providing more information than a selected histological section taken by biopsy [30]. It also avoids tissue damage and alteration of cellular architecture which may occur in the biopsy and histological preparation [4]. This noninvasive technique also helps to direct the site of biopsy by identifying areas with cellular atypia and thus avoiding the need for multiple biopsies. Subsequently, this results in a dramatic improvement of the diagnostic yield of the biopsy [32]. Other potential roles of CE include the rapid diagnosis of benign and malignant mucosal lesions in an outpatient or operating room setting, surveillance, guided biopsies, and intraoperative evaluation of tumor resection margins.

Despite its advantages, CE does have its limitations. Most notably, CE can only evaluate the most superficial cell layer of the mucosal epithelium. This is most likely due to a number of factors including (i) poor penetration of methylene blue which only stains a few superficial layers, (ii) short focal distance of the scope (i.e., CE can only assess to a depth of 80 um at 60x magnification and 30 um at 150x magnification), and (iii) optical artifact at high magnification due to glare from light reflected from cells not in focus. Subsequently, assessment of submucosal lesions or lesions occupying deeper cell layers becomes more difficult [4, 27, 28, 30, 32, 33].

The lack of depth of penetration prevents the evaluation of important histological information especially when vertical extent of dysplasia is crucial in distinguishing the different grades of dysplasia from carcinoma in situ and invasive carcinoma. As a result, these factors could affect the sensitivity of CE, thus accounting for some of the false negative diagnostic results noted by authors. The potential impact of CE missing a malignant lesion needs to be taken into consideration if this technology is to one day substitute histopathology. Future investigation into better penetrating dyes, advances in digital optics, and image enhancements will eventually allow better vertical staining and increased resolution of the deeper cell layers which would translate CE in becoming a much more sensitive and accurate diagnostic tool [31].

A pilot study conducted at our institution also investigated some of the limitations and potential advantages of CE in the evaluation of head and neck mucosal lesions. From our preliminary experience, technical difficulties with line of sight, access difficulties to mucosal surfaces, scope positioning, and problems with consistent image quality due to artifact were consistent with those found by previous authors [30, 33].

Our pilot study also demonstrated that although CE is a simple, rapid, repeatable, noninvasive examination performed with standard equipment, there is a learning curve associated with its use. However, once one is accustomed with this detection system, CE can be performed almost as quickly as an outpatient flexible fiberoptic nasopharyngoscopic examination.

In conclusion, *in vivo* assessment of head and neck mucosal pathology may be applied to (i) early detection of premalignant and malignant lesions, (ii) serial follow-up examinations of suspicious lesions such as leukoplakia and lichen planus, and (iii) assessment of resection margins. Despite its limitations, CE represents a promising optical technology that may afford reliable, accurate, and noninvasive *in vivo* assessment of cytological pathology. Prospective investigation with CE is currently ongoing at our institution and necessitates close collaboration between otolaryngologists and pathologists. We hypothesize that future study will demonstrate improved sensitivity, specificity, and accuracy of contact endoscopy in the diagnosis of head and neck tumors.

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Review Article

ING Genes Work as Tumor Suppressor Genes in the Carcinogenesis of Head and Neck Squamous Cell Carcinoma

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world. The evolution and progression of HNSCC are considered to result from multiple stepwise alterations of cellular and molecular pathways in squamous epithelium. Recently, inhibitor of growth gene (*ING*) family consisting of five genes, *ING1* to *ING5*, was identified as a new tumor suppressor gene family that was implicated in the downregulation of cell cycle and chromatin remodeling. In contrast, it has been shown that *ING1* and *ING2* play an oncogenic role in some cancers, this situation being similar to TGF-β. In HNSCC, the *ING* family has been reported to be downregulated, and ING translocation from the nucleus to the cytoplasm may be a critical event for carcinogenesis. In this paper, we describe our recent results and briefly summarize current knowledge regarding the biologic functions of *ING* in HNSCC.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world. More than 500,000 new cases and the over 50% mortality rate annually indicate a major health problem worldwide [1]. HNSCC is a broad term that represents squamous cell carcinomas that arise in the upper aero- and digestive tract, including the larynx, the pharynx, and the oral cavity. These sites form a functional and anatomic unit and share exposure to the same etiological factors in carcinogenesis [1]. It is well known that smoking and alcohol abuse are major risk factors for HNSCC. Additionally, human papillomavirus (HPV) infection is another implicated risk factor, in particular for oropharyngeal SCC [2, 3].

The evolution and progression of HNSCC are considered to result from multiple stepwise alterations of cellular and molecular pathways in the squamous epithelium [4]. Although lifestyle factors account for the majority of HNSCCs, genetic alterations will cause some individuals to be more sensitive to these environmental factors. Therefore, screening for reliable genetic changes can provide a possible opportunity to predict the risk of malignant transformation. Tumor suppressor genes (TSGs) are often referred to as "gatekeepers" because they prevent cancer development by direct control of cell growth through genes such as *p53* and *p16,* the inactivation of which has been reported in many tumors. The alterations of TSG, including mutation, loss of heterozygosity (LOH), and microsatellite instability, are considered to increase genetic susceptibility for malignant transformation. Previous studies have identified that alterations of *p53* and *p16* are associated with the development and progression of HNSCC [5–8]. Inhibitor of growth gene (*ING*) family, a new candidate TSG class, is implicated in cell cycle control, senescence, apoptosis, DNA repair, and chromatin modeling. The loss or downregulation of *ING* expression has been observed in HNSCC. In this paper, we summarized current knowledge on the biological function of *ING* family members and their status in the tumorigenesis of HNSCC.

2. ING Gene Family

ING1, the first member of the *ING* family, was discovered through a subtractive hybridization assay between normal mammary epithelium and breast cancer cell lines and was

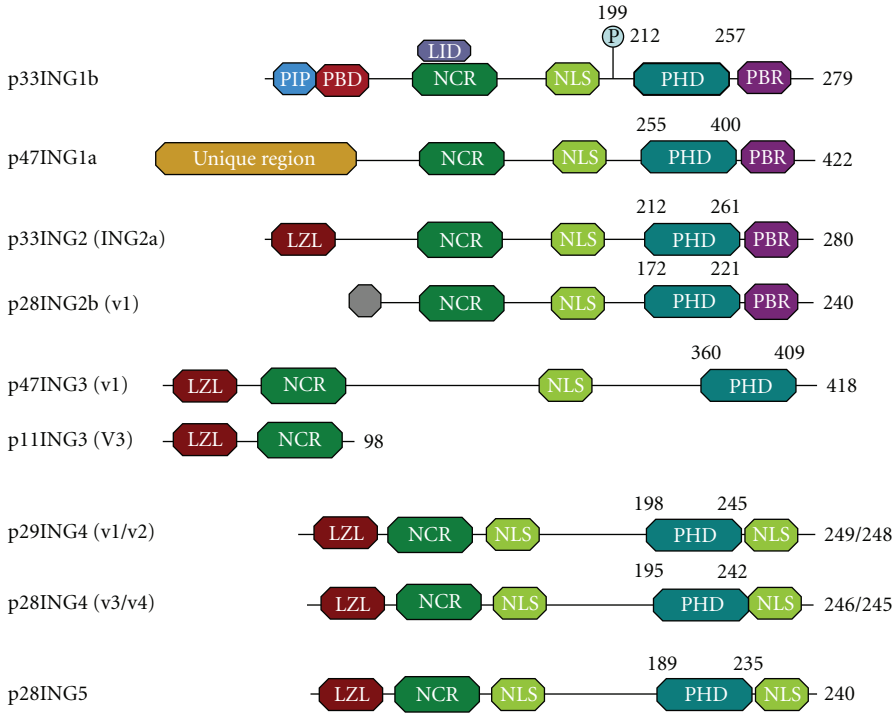

Figure 1: Structure of ING proteins in *Homo sapiens*. Each ING protein with its name and encoding major variants is listed on the left. The characterized domain composition, approximate location, is shown on the right. All ING proteins contain three conserved regions, a PHD (plant homeodomain), NLS (nuclear localization signal), and NCR (novel conserved region) from C-terminal region to N-terminal region. An LZL (leucine zipper-like domain) is present in ING2-5. $p33^{ING1b}$ also have a PIP (PCNA-Interacting Protein Motif) domain through which it binds to PCNA following UV irradiation, a PBD (partial bromodomain) which commonly found in chromatin-associated protein, and an LID (Lamin Interaction domain). $p33^{ING1b}$ binds to lamin A/HDAC complexes via LID to maintain its levels and biological function in nucleus. Additionally, phosphorylation sites were found at serine 199 of $p33^{ING1b}$. 14-3-3 bind to phosphorylated serine 199 result in translocation of $p33^{ING1b}$ from the nucleus to the cytoplasm.

shown to play a role in neoplastic transformation [9]. Subsequently, four other members of *ING* family, *ING2*, *ING3*, *ING4*, and *ING5*, were identified by computer homology searches and were shown to have 32 to 76% DNA sequence homology with *ING1* [10–13]. The *ING* genes each mapped to independent chromosomes: 13q34, 4q35, 7q31, 12p13.3, and 2q37.3. All of the *ING* genes except *ING3* localize to the subtelomeric region of their respective chromosomes [14]. In addition, phylogenetic analysis identified that *ING* genes are conserved in many species, including humans, mice, rats, and yeast [15]. Alignment data show that the human and mouse ING1 and ING3 proteins are 90% identical, whereas the human and frog ING1 and ING3 proteins are 81% and 82% identical, respectively [16]. These data suggest that *ING* genes play important roles in biological processes central to life.

Most *ING* genes, excluding *ING5*, encode variants due to different promoters, exons, and alternative splice variants. ING1 encodes four isoforms, $p47^{ING1a}$, $p33^{ING1b}$, $p24^{ING1c}$, and $p27^{ING1d}$, which vary in mass between 24 and 47 kDa. Among these isoforms, $p33^{ING1b}$ is the most widely expressed in normal tissues [17]. ING2 encodes two isoforms. ING2a, also called ING2 and ING1L, encodes a 280-aa protein ($p33^{ING2}$) that shares 58.9% similarity with $p33^{ING1b}$ [10, 11]. Recently, ING2b was identified and shown to be transcribed from the middle of intron 1 of ING2a [18]. In addition, ING3 encodes two isoforms, $p47^{ING3}$ and $p11^{ING3}$ [12]. ING4 encodes eight splice variants: ING4_v1, v2, v3, v4, ING4ΔEx2, ΔEx3, ΔEx6A, and ΔEx6B [19, 20]. Only ING5 encodes a unique 240-aa protein ($p28^{ING5}$) [13]. Splice variants of ING proteins may compensate or compete with each other and create more diversity in ING functions.

3. The Structure and Function of ING Proteins

All ING proteins contain a plant homeodomain (PHD) in the C-terminal region, a nuclear localization signal (NLS), and a domain with an unknown function called the novel conserved region (NCR) (Figure 1). The N-terminal region of each ING protein is unique, which determines the differential structures of ING proteins [21]. The PHD domain, a zinc finger domain that binds histone H3 in a methylation-sensitive manner, has been implicated in chromatin remodeling [22]. Localization of ING proteins in the nucleus is critical to their function [23]. The NLS targets ING1 or other ING proteins to different chromatin domains in the nucleus and nucleolus in response to UV-induced DNA damage [24]. Moreover, a leucine zipper-like (LZL) domain is present in ING2–5 and has the potential to form a

hydrophobic face near the N-terminus of ING proteins [25]. Regarding its function, the LZL domain may be linked to nucleotide excision repair and induction of apoptosis [26]. $p33^{ING1b}$ also carries three other domains. A proliferating cell nuclear antigen-(PCNA-) interacting protein motif (PIP) domain binds with PCNA following UV irradiation. A partial bromodomain (PBD) is commonly found in chromatin-associated protein. A lamin interaction domain (LID) binds with lamin A/HDAC complexes to maintain its levels and biological function in the nucleus [27]. Recently, a phosphorylation site was found at serine 199 of $p33^{ING1b}$. 14-3-3 family proteins can bind to phosphorylated serine 199, resulting in translocation of $p33^{ING1b}$ from the nucleus to the cytoplasm [28].

4. PHD Domain and Epigenetic Control

Although the *ING1* gene was cloned as a candidate gene for tumor suppression, studies on the effects of overexpression or downregulation of ING family proteins on various cellular processes imply that the roles of the *ING* family genes in tumorigenesis depend on cellular contexts; they could also function as oncogenes in several aspects [29]. Therefore, we first described the functions of ING family proteins in the epigenetic control of gene transcription and DNA replication, details of which are now going to be elucidated.

Epigenetic control of gene transcription is attained partly by modulation of covalent modifications such as acetylation, methylation, and/or phosphorylation of nucleosomal histones within gene promoters [30]. ING proteins are known to be a component of either histone acetylase (HAT) complexes or histone deacetylase (HDAC) complexes that activate and inactivate gene transcription, respectively. $p33^{ING1b}$ interacts with the mSin3/HDAC complex and also with proteins associated with HAT activity such as p300, inducing hyperacetylation of histones H3 and H4 [31–33]. Similarly, ING2 complex with p300 also serves as a component of the mSin3/HDAC complex [34–36]. ING3 associates with the hNuA4/Tip60 HAT complex (nucleosome acetyltransferase of H4 and Tat interactive protein, respectively; Tip60 is the human homolog of yeast Esa1 HAT) that is responsible for acetylation of histone H4 and H2A [36, 37]. Both ING4 and ING5 bind to p300 [13], but they also associate with different HAT complexes. ING4 is identified as a component of a four-subunit HAT complex containing HBO1 (histone acetyltransferase binding to origin recognition complex-1). HAT and its cofactors JADE1/2/3 preferentially acetylate histone H4 [36]. ING5 associates with MOZ (monocytic leukemic zinc-finger protein)/MORF (MOZ related factor) HAT and its cofactor BRPF (bromodomain-PHD finger protein) 1/2/3, resulting in increased specificity for acetylation of histone H3 lysine 14 [36].

In turn, the PHD domains of ING family proteins were recently shown to recognize trimethylated lysine 4 of histone H3 (H3K4me3) that in many cases associates with active gene transcription [30]. ING2 was firstly shown to bind H3K4me3 via its PHD domain and stabilize the mSin3-HDAC complex, resulting in repression of DNA damage-induced transcription of cyclin D1 gene [22, 38]. ING1 also binds H3K4me3, and this binding is somehow necessary for ING1-mediated DNA repair upon UV irradiation as well as doxorubicin-mediated induction of apoptosis in HT1080 fibrosarcoma cells [39]. Intriguingly, cancer-associated mutations in the ING1 PHD domain impaired the binding of ING1 to H3K4me3 with concomitant loss of functions in DNA repair and apoptosis, implying that the binding of ING1 to H3K4me3 underlies its tumor-suppressive functions [39]. Binding of ING4 to H3K4me3 and its biological outcomes were extensively studied [40–42]. Promoters bound by ING4 in response to DNA damage were identified using a chromatin immunoprecipitation technique followed by whole genome promoter tiling arrays [42]. ING4 was recruited to its target promoters upon interaction with H3K4me3 and increased the acetylation of histone H3 lysine 9, leading to activation of gene transcription and sensitization to cell death or inhibition of anchorage-independent cell growth [42].

These compiled lines of evidence indicate that one basic function of ING family proteins is to translate H3K4me3 markings on the nucleosomes into activation or inactivation of gene transcription, DNA replication, or DNA repair through the associated HAT or HDAC complex. Based on this simple framework, further questions as follows may be posed for the elucidation of the substance of "cellular contexts" as described above, the same family member of ING can complex with either HAT or HDAC. (1) What determines the combination of ING proteins and HAT or HDAC complexes and the final outcomes? (2) What modulates the inducible or constitutive binding of ING proteins to H3K4me3 within gene promoters? (3) Do ING family members compete for the binding to the same H3K4me3 within a promoter or have some specificity for it? To answer these questions, identification of the genes modulated by ING family proteins and side-by-side analyses of transcriptional response of the gene and factors that associate with ING proteins as made in [42] may be helpful.

5. ING and DNA Repair

The balance between cell growth and cell death is characterized in tissue development and homeostasis. In response to slightly stressful stimuli, cells usually start a cellular stress response including DNA repair to ensure survival. However, when irreversible damage accumulates, cells can permanently arrest the cell cycle (cellular senescence) or trigger a cell death program (apoptosis) [43].

ING1 is the founding member of the ING family and the most well studied. Paul et al. confirmed that ING1 interacts specifically with three proteins, p38MAPK, mammalian JNK/p38MAP kinase (MEKK4), and RAD50, by utilizing a cross-species (yeast, fly, and human) bioinformatics-based approach. Both p38MAPK and MEKK4 participate in a well-defined stress response pathway. These novel ING-interacting proteins further link ING proteins to cellular stress and DNA damage signaling [44]. Nucleotide excision repair (NER) is a crucial stress response mechanism for

maintaining genomic stability. Overexpression of $p33^{ING1b}$ can enhance NER of both UV-damaged genomic DNA and exogenous plasmid DNA in a host-cell-reactivation assay. Moreover, $p33^{ING1b}$ requires the participation of functional p53 in DNA repair and may be a crucial component in the GADD45-mediated NER pathway [45]. Conversely, missense mutations in the SAP30-interacting domain and PHD finger motif of *ING1* abrogated the enhancement of NER in a host-cell-reactivation assay and a radioimmunoassay [46]. In addition, PCNA is an essential processivity factor for DNA polymerases and functions in both eukaryotic chromosomal DNA replication and NER. $p33^{ING1b}$ contains a PIP motif within its N-terminus. By competitively binding PCNA through its PIP domain, $p33^{ING1b}$ may contribute to regulating the switch from DNA replication to DNA repair [47]. In both normal human epithelial keratinocytes (NHEKs) and a keratinocyte cell line, HaCaT, the expression levels of $p33^{ING1b}$ were elevated by UV induction independent of p53 status, thus suggesting that ING1 may participate in the cellular stress response and skin carcinogenesis [48]. In addition, ING2 interacted with certain HAT or HDAC proteins through its LZL domain, instead of the PHD region, to regulate histone H4 acetylation, chromatin decondensation, and NER [35]. Therefore, ING proteins may participate in DNA repair through the regulation of the NER pathway in response to cellular stress and DNA damage.

6. ING Proteins and Cell Cycle

Loss of proper control of the cell cycle is a major cause of cell transformation. Cellular senescence refers to the arrest in the G0 phase of the cell cycle [49]. $p33^{ING1b}$ was upregulated in senescent human fibroblasts, and antisense $p33^{ING1b}$ extends the proliferative lifespan of normal human fibroblasts [50]. Moreover, ectopic expression of ING1 in diploid human fibroblasts resulted in cell cycle arrest with some features of cellular senescence [51]. Chromatin immunoprecipitation analysis indicated that the chromatin binding affinity of $p33^{ING1b}$ was higher in senescent cells compared with young cells, thus suggesting that ING1-mediated functions may be subject to age-dependent mechanisms of control directed to prevent induction of apoptosis in senescent but not in young cells [52]. ING2 enhanced the interaction between p53 and p300 and acted as a cofactor for p300-mediated p53 acetylation. Overexpression of ING2 induced senescence in young fibroblasts in a p53-dependent manner. Conversely, the downregulation of ING2 expression by siRNA transfection led to delaying the onset of senescence [53].

Previous research has demonstrated that overexpression of $p33^{ING1b}$ increased the number of human diploid fibroblasts in the G0/G1 phase. Conversely, antisense $p33^{ING1b}$ permitted these cells to enter S phase [9]. Cyclin E is a member of the cyclin family and binds to Cdk2 in the G1 phase, which is required for the transition from G1 to S phase. Expression of $p33^{ING1b}$ in human hepatocellular carcinoma (HCC) was inversely correlated with cyclin E kinase activity by autoradiography [54], thus implicating that the reduction of $p33^{ING1b}$ expression may contribute to the process of malignant transformation of HCC via an increase of cyclin E kinase activity. Another study indicated that ectopic expression of ING1b in H1299 cells sensitized the cells to short-term G2/M cell cycle delay [55]. In addition, adenovirus-mediated overexpression of ING1 in mouse mammary epithelial cells resulted in the downregulation of cyclin B1, which accumulates during the G2-M phase of the cell cycle [55]. Moreover, adenovirus-ING4-mediated transfection of PANC-1 human pancreatic carcinoma cells inhibited cell growth, altered the cell cycle with S-phase reduction and G2/M phase arrest, and induced apoptosis [56]. These findings suggest that ING may regulate cell senescence and cell cycle via the G1/S and the G2/M cell cycle checkpoints.

7. ING and Apoptosis

Apoptosis plays important roles in normal development and removal of the cells carrying severe DNA-damages induced by DNA damaging agents. In cancer cells, activation of pathways that favor cell survival instead of apoptosis may contribute to tumorigenesis. Many different agents and growth environmental factors can be used to induce apoptosis, such as cytotoxic drugs, irradiation, and serum starvation. Expression of ING1 increased upon the induction of apoptosis in P19 mouse teratocarcinoma cells by serum deprivation. Elevated expression of ING1 cooperated with c-myc gene expression to enhance the extent of apoptosis in P19 and rodent fibroblast cells [57]. Ectopic expression of $p33^{ING1b}$ also sensitized cells to apoptosis induced by etoposide, taxol, and doxorubicin [24, 52, 55]. Ectopic expression of $p33^{ING1b}$, but not $p47^{ING1a}$, significantly enhanced UV- or hydrogen peroxide-induced apoptosis in young (low passage) but not senescent Hs68 cells. Moreover, cotransfection of $p33^{ING1b}$ and p53 increased the percentage of apoptotic cells compared to transfection of either of these two proteins alone [52]. Conversely, expression of $p33^{ING1b}$ antisense constructs protects cells against apoptosis [57] and promotes neoplastic transformation [9]. $p33^{ING1b}$ activates transcription of the p21/WAF1 promoter, a key mechanism required for p53-mediated cell growth control [58]. Adenovirus-mediated transfer of $p33^{ING1b}$ with p53 suggested an additive or synergistic effect on apoptosis in immortal human cancer cells [59]. In addition, $p33^{ING1b}$ was demonstrated to influence tumor necrosis factor (TNF)-α-mediated apoptosis in Hs68 cell by upregulation of HSP70 expression and enhancement of the ability of TNF-α [60]. All ING proteins tested to date show the ability to regulate apoptosis in varying degrees through similar or different signal pathways. For example, the ING2 PHD finger interacts with phosphatidylinositol 5-phosphate (PtdIns5P) in vivo, and their interaction regulates the ability of ING2 to activate p53 and p53-dependent apoptotic pathways [61]. Increased ING2 expression was also found to increase Bax expression and enhance UVB-induced apoptosis in human melanoma cells [62]. Additionally, overexpression of ING3 significantly promoted UV-induced apoptosis through the activation of the Fas/caspase-8 pathway, and knockdown of ING3

remarkably decreased UV-induced apoptosis [63]. These results suggested that ING might induce apoptosis through varied pathways in response to different agents.

8. ING and p53

p53 is an important TSG that is inactivated in many cancers. p53 assimilates disparate input signals, including oncogene activation, DNA damage, mitotic impairment, and oxidative stress, to initiate appropriate outputs such as initiation of DNA repair, cell cycle arrest, senescence, or apoptosis [64]. The physical and functional interactions between ING and p53 have been investigated widely, but the conclusions are not consistent. In overexpression experiments, all ING proteins except ING3 have been observed to coimmunoprecipate with p53. Moreover, ING-induced cell cycle arrest and apoptosis were compromised in p53-deficient cultured cells [25, 65, 66]. Functional p53 is required for $p33^{ING1b}$-mediated inhibition of cell growth in cultured cells. Furthermore, $p33^{ING1b}$ was proposed to compete with murine double minute 2 (MDM2), an important negative regulator of p53, for the same binding site on p53, leading to an increase in the stability and activity of p53 [67]. ING2 may modulate p53-dependent chromatin remodeling, apoptosis, and DNA repair by functioning as a scaffold protein to mediate the interaction between p53 and p300 [35]. Additionally, overexpression of ING4 or ING5 leads to a reduction in colony-forming efficiency, inhibition of S-phase, and induction of apoptosis in a p53-dependent manner. ING4 and ING5 may stabilize p53 and enhance p53-mediated cellular responses to genotoxic stresses and apoptotic stimuli through ING4/5-mediated acetylation of p53 [13]. These results implicated that ING proteins may be significant modulators of p53 function. However, it was worth noting that the experiments of *ING1* knockout mice and knockout cells indicated that ING1 functions were mostly independent from the p53 signaling pathway in physiological conditions [68, 69]. In mice, the *Ing1* gene decodes three spliced isoforms. Ing1a and Ing1c encode a 31 kDa protein, and Inglb encodes a 37 kDa protein ($p33^{ING1b}$ in human). Loss of $p37^{Ing1}$ induced BAX expression and increased DNA damage-induced apoptosis in primary cells and mice irrespective of p53 status. Moreover, p53 functions are unperturbed in $p37^{Ing1}$-deficient cells. Moreover, $p37^{Ing1}$ suppressed the formation of spontaneous follicular B-cell lymphomas in mice. Therefore, $p37^{Ing1}$ can negatively regulate cell growth, apoptosis, and tumorigenesis in a p53-independent manner [69]. Previous studies also demonstrated that the expression of ING might be independent on p53 status in some tumor tissues. Decreased ING1 expression may play important roles in tumorigenesis of the specimens with expression of the wild-type p53 gene in gastric carcinoma [70] and nonsmall cell lung carcinoma (NSCLC) [71].

In addition, ING proteins are found to function in a p53-independent manner. One major p53-independent function of ING proteins may be negative regulation of NF-κB. $p33^{ING1b}$ and ING2 proteins were found to suppress expression of NF-κB by upregulating HSP70 gene expression and augment TNF-α-induced apoptosis [60]. ING4 is shown to directly interact with p65(RelA) in glioma cells to inhibit transcriptional activity of NF-κB. Correspondingly, the expression of NF-κB-responsive genes is shown to be significantly increased in ING4 knockdown cells [72]. Another study showed that ING4 suppresses NF-κB-regulated promoters by binding with both of p65 and H3K4me3 on the promoter [73]. This recruitment of ING4 accompanies the reduction of p65 phosphorylation and concomitant change of complex formation of p65 with p300 (HAT) to HDAC1 resulting in the decrease of acetylated histones and H3K4me3 within the promoter [73]. Additionally, ING4 was found to affect the stability of hypoxia inducible factor (HIF) and mediate HIF activity [72].

Based on these findings, ING and p53 may function independently in apoptosis pathways, but they can influence the activity of each other in tumorigenesis [15]. As epigenetic regulators of chromatin structure, ING proteins may amplify the effects of p53 on gene expression and also directly affect DNA repair and apoptosis independently of p53 by altering chromatin structure.

9. ING Genes and Tumorigenesis

Previous studies have implicated members of the *ING* family as candidate type II TSGs that are involved in a variety of processes, including DNA repair, cell cycle control, senescence, apoptosis, and chromatin remodeling, which are critical points for genomic integrity and stability (Figure 2). Thus, loss or decrease of ING expression may be a potential key point in tumorigenesis. Knockout experiments demonstrated that ing1-dificient mice were more sensitive to total body gamma radiation, and loss of ing1 was associated with earlier onset and higher incidence of lymphomas [68].

Loss of nuclear $p33^{ING1b}$ was observed in melanoma, seminoma, papillary thyroid carcinoma, ductal breast carcinoma, and acute lymphoblastic leukemia by comparing these neoplastic tissues with normal cells and tissues [74]. Until now, inactivation and reduced expression of *ING* genes has been reported in cancers of lung [71], breast [75], stomach [70, 76], esophagus [77], blood [78], brain [79], and HNSCC [80]. Interestingly, ING gene mutation is uncommon in cancer. In fact, translocation of ING proteins from the nucleus to the cytoplasm has been observed in some types of cancer, such as the tumors of the breast [75] and brain [79], melanoma [74], and lymphoblastic leukemia [78]. Therefore, the ING cellular compartment shift from the nucleus to the cytoplasm may cause loss of normal cellular function and may play a central role in tumorigenesis and progression.

Like other ING genes, nonphysiological overexpression of ING2 induces apoptosis and cell cycle arrest via p53 modification [10], and decreased ING2 expression was found in cutaneous cancer [81] and HNSCC [82]. However, expression of ING2 was upregulated in colorectal cancer [83], Burkitt's lymphoma, and cervical cancer [29]. Moreover, ING2 may bind to the RPB1-mSin3A-HDAC complex on the

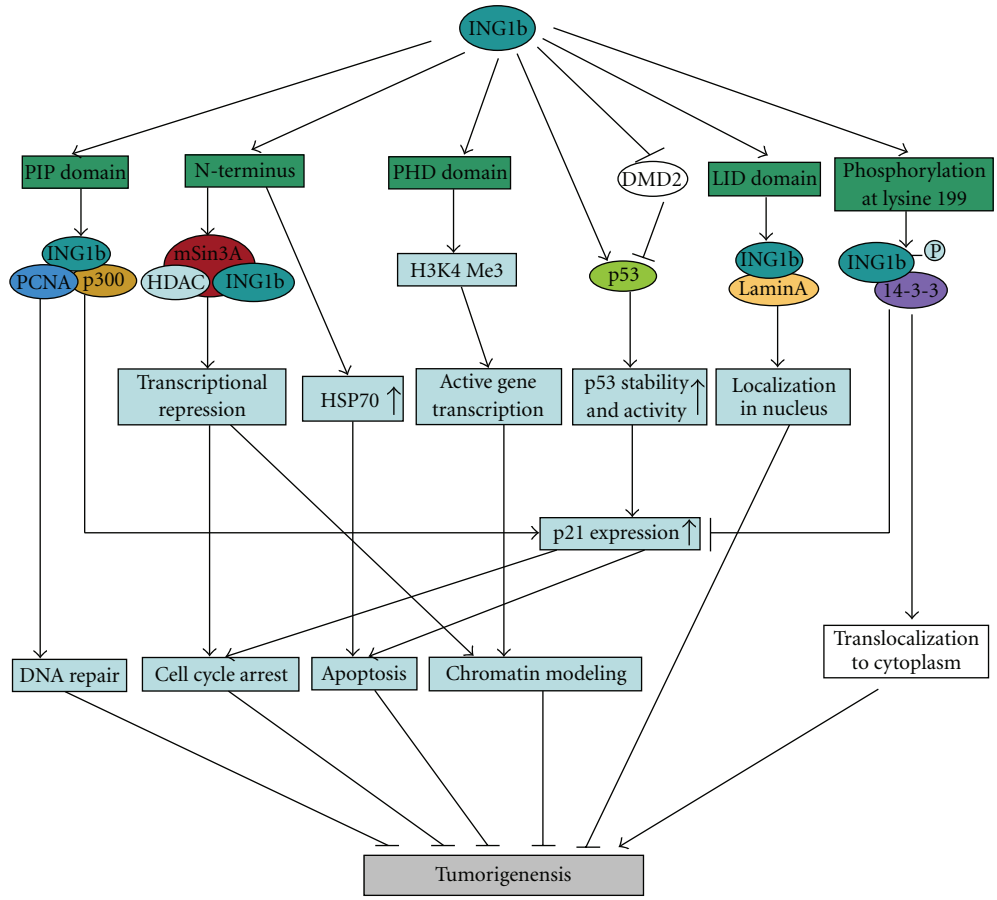

Figure 2: The role of $p33^{ING1b}$ protein in tumor supression. $p33^{ING1b}$ could recognize trimethylated lysine 4 of histone H3 (H3K4me3) by PHD domain and has been implicated in chromatin remodeling and activation of some genes transcription. This binding is somehow necessary for induction of DNA repair and cell death. $p33^{ING1b}$ also associates with the Sin3/HDAC-mediated transcriptional repression through its unique N-terminal sequence and may be involved in repression of some essential cell cycle regulator genes. Moreover, $p33^{ING1b}$ binds PCNA and p300 complex to promote DNA repair through a PIP motif in response to UV-irradiation and, subsequently, may trigger apoptosis by the induction of p21 expression. $p33^{ING1b}$ competes with murine double minute 2 (MDM2) leading to an increase in the stability and activity of p53. p21, the one of the targets of p53, is also upregulated to involve in cell cycle arrest and the induction of apoptosis. Additionally, $p33^{ING1b}$ could upregulate expression of HSP70 gene to induce apoptosis independently of p53 status. Furthermore, $p33^{ING1b}$ binds to lamin A via LID domain to stabilize its level and biological function in nucleus. Conversely, 14-3-3 can bind to $p33^{ING1b}$ with phosphorylated serine 199 and results in translocation of $p33^{ING1b}$ from the nucleus to the cytoplasm, which may involve in tumorigenesis.

MMP13 promoter to upregulate MMP-13 expression [83]. Thus, the function of ING2 may be different depending on the cancer type. A recent study suggested that ING2 is a novel mediator of transforming growth factor (TGF)-β-dependent responses in epithelial cells [84]. TGF-β is considered to have tumor suppressor-like functions in normal epithelium and also have oncogenic functions in invasive metastatic cancers. Therefore, ING2 may play different roles in normal cells and cancers by mediating the TGF-β signaling pathway.

10. Expression of ING Genes in HNSCC

Previous studies have demonstrated 45.5%–68% LOH of *ING* genes in HNSCC (Table 1), and 50%–76% decreases in the mRNA levels of *ING3–5*. In recent studies, we also investigated expression as well as the subcellular localization of ING proteins in 214 cases of HNSCC by immunohistochemistry. Decreased expression of $p33^{ING1b}$, ING4, and ING5 in nuclei was observed in 36.9%, 61.3%, and 36% of the HNSCC cases, respectively. These results suggest that the loss or downregulation of nuclear expression of ING proteins participates in tumorigenesis of HNSCC. By contrast, mutations of the *ING* genes are rare (0–4.3%) in HNSCC although most of the mutations are present in the domains critical for the functions of ING proteins (Table 1), suggesting that mutations are not the major cause for *ING* family inactivation. In addition, the shift of $p33^{ING1b}$ from the nucleus to the cytoplasm was observed in 24.5% of 49 in oral SCCs [85]. In our studies, aberrant cytoplasmic expression of $p33^{ING1b}$, ING4, and ING5 was detected in 14.5%, 68.8%, and 47.7% in 214 cases of HNSCC, respectively [80, 86, 87], while no or seldom cytoplasmic expression of these ING proteins was detectable in the cases of normal mucosa.

Table 1: *ING* gene mutation and expression in HNSCC.

ING	Origin	Methods	Mutation type/expression change	Position	Frequency	Reference
ING1	Patient	MM	LOH	13q34	20/44(45.5%)	[88]
	Cell lines	Sequencing	No mutation		0/5	
	Patient	Sequencing	No mutation		0/20	
	Patient	MM	LOH	13q33-34	23/34(68%)	[89]
	Patient	PCR-SSCP	Missense	PHD (215)	1/23(4.3%)	
	Patient	PCR-SSCP	Missense	PHD (216)	1/23(4.3%)	
	Patient	PCR-SSCP	Missense	NLS (192)	1/23(4.3%)	
	Patient	RT-PCR	Downregulation		6/12(50%)	[90]
	Patient	IHC	Downregulation		37/49(76%)	[85]
	Cell lines	Sequencing	No mutation		0/3	[86]
	Patient	IHC	Downregulation		79/214(36.9%)	
ING2	Patient	MM	LOH	4q35.1	33/55(54.6%)	[82]
ING3	Patient	MM	LOH	7q31	22/46(48%)	[91]
	Patient	RT-PCR	Downregulation		20/40(50%)	
	Patient	PCR-SSCP	Missense	LZL(20)	1/49(2%)	
	Patient	RT-PCR	Downregulation		37/71(52.1%)	[92]
	Patient	RT-PCR	Upregulation		15/71(21%)	
ING4	Patient	MM	LOH	12p13	33/50(66%)	[87]
	Patient	Sequencing	No mutation		0/50	
	Patient	Q-PCR	Downregulation		38/50(76%)	
	Patient	Q-PCR	Upregulation		7/50(14%)	
	Cell lines	Sequencing	No mutation		0/3	[93]
	Patient	IHC	Downregulation		96/214(44.9%)	
ING5	Patient	RT-PCR	Downregulation		19/31(61.3%)	[94]
	Patient	Sequencing	Missense	LZL(33)	1/31(3.2%)	
	Patient	Sequencing	Missense	NCR(68)	1/31(3.2%)	
	Patient	Sequencing	Missense	NCR(74)	1/31(3.2%)	
	Cell lines	Sequencing	No mutation		0/3	[80]
	Patient	IHC	Downregulation		77/214(36%)	

Note: MM, Microsatellite marker; PCR-SSCP, Polymerase chain reaction-single strand conformation polymorphism; RT-PCR, Retrotranscription-polymerase chain reaction; Q-PCR, Quantitative-polymerase chain reaction; IHC, immunohistochemistry.

Nuclear localization of ING proteins is required for their normal function. Therefore, decreased nuclear expression of ING proteins, through either downregulation of nuclear expression or relocation from the nucleus to cytoplasm, may play a crucial role in the development and progression of HNSCC (Figure 3) and may be a new biomarker for the tumorigenesis of HNSCC.

The mechanism of translocation of ING proteins is not fully understood. Recently, a study from Riabowol's group demonstrated that $p33^{ING1b}$ can especially bind members of the 14-3-3 family through phosphorylation at serine residue 199 [28]. 14-3-3 family members primarily reside in the cytoplasm and are associated with phosphorylated ligands involved in many cellular processes, including regulation of the cell cycle and DNA damage

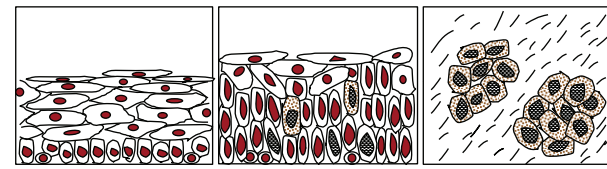

Figure 3: The schematic diagram of ING proteins expression in the malignant development of HNSCC. Nuclear expression of ING proteins is downregulated from normal squamous epithelium to dysplastic epithelium and invasive HNSCC. In contrast, the cytoplasmic expression of ING proteins in dysplastic epithelium and invasive HNSCC is gradually increased compared with normal squamous epithelium. The positive expression of ING proteins is shown with brown color.

checkpoints [95]. 14-3-3 binding results in tethering of significant amounts of $p33^{ING1b}$ in the cytoplasm [28]. Additionally, cytoplasmic $p33^{ING1b}$ could be imported into the nucleus through interactions between its intrinsic NLS and karyopherins $\alpha 2$ and $\beta 1$ [96]. In the nucleus, lamin A binds and targets ING1 and regulates ING1 levels and biological function [27]. Therefore, 14-3-3, karyopherins $\alpha 2$ and $\beta 1$, and lamin A are involved in the dynamic regulation of subcellular distribution of ING1. Recently, we investigated the expression of $p33^{ING1b}$ and 14-3-3η in 214 cases of HNSCC by immunohistochemistry and found that cytoplasmic $p33^{ING1b}$ expression was significantly associated with 14-3-3η expression. Moreover, double immunofluorescence results confirmed the coexpression of $p33^{ING1b}$ and 14-3-3η (unpublished data). These data indicated that 14-3-3η plays an important role in the cytoplasmic accumulation of $p33^{ING1b}$ in HNSCC. However, the function of cytoplasmic ING is unclear and needs to be further studied.

There have been a few studies on the correlation between clinicopathological variables and expression of the *ING* genes. High LOH frequency of ING2 was statistically associated with advanced T stage, suggesting that ING2 LOH might occur at the late stage of HNSCC progression [82]. Although no clinicopathological variables were significantly related to the levels of ING3 mRNA, decreased expression of ING3 mRNA was associated with high mortality and was an independent prognostic factor for poor overall survival [92]. In our recent studies, no significant correlation was found between high nuclear expression of $p33^{ING1b}$ and clinicopathological variables in HNSCC, but high expression of cytoplasmic $p33^{ING1b}$ was significantly correlated with poor differentiation, T staging, lymph node metastasis, and TNM staging [86]. Also, high expression of nuclear ING4 in HNSCC was negatively correlated with poor differentiation, T staging, and TNM staging, while high expression of cytoplasmic ING4 in HNSCC was positively correlated with lymph node metastasis [93]. Also in the case of ING5, its nuclear expression correlated with differentiation of HNSCC, and abundant cytoplasmic expression correlated with poor differentiation [80].

11. Conclusions

The *ING* family genes are supposed to belong to type II TSG and are involved in multiple cellular processes including chromatin remodeling, DNA repair, cell cycle control, senescence, and apoptosis. ING proteins are expressed independently of p53 status and function in both p53-dependent and p53-independent manner. Loss or downregulation of *ING* genes expression and/or translocation of ING proteins from the nucleus to the cytoplasm may play an important role in neoplastic development of HNSCC. Thus, the *ING* gene family could be a novel p53-independent biomarker for HNSCC. Further elucidation of the functions of ING family proteins, which can be either tumor suppressive or tumorigenic, will rationalize their application for a biomarker, and it will also reveal the potentiality of ING proteins as the therapeutic target [97].

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