Chapter 2

Molecular Events in Tobacco Related Oral Cavity Cancers

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Abstract

Oral carcinogenesis is a multistep process involving numerous molecular events during the initiation, promotion and progression of the disease. The present review summarizes along with important reports of several molecular signatures that could be distinctly correlated with susceptibility, progression and invasion/metastasis of oral cancer. In furtherance the data and views are presented to translate the wisdom to develop salivary based biomarkers for its applicability in clinical oncology. The review documents the role of tobacco, dietary factors, reactive oxygen species, nitrates, nitrites, antioxidant enzymes, genetic susceptibility, polymorphism and mutations in $p53$ in oral cancer development. Altered glycosylation of cell membrane proteins is critically important in several stages of oral carcinogenesis process and has been associated with all hallmark capabilities.

Overall, the data on nutritional status, genetic susceptibility may be useful to derive risk profiles for healthy individuals as well as patients and to design effective and specific preventive strategies. The markers of progression and metastasis might be helpful in early detection, prognostication, treatment monitoring and may also serve as important drug targets for future interventions. Saliva can prove to be an effective non-invasive tool for assessing biomarkers with its easy applicability in clinical set-up.
Cancer arises as a result of multiple molecular events that develop from the combined influences of an individual's genetic predisposition and exposure to environmental carcinogens. Chronic exposure to carcinogens such as tobacco and alcohol can damage individual genes as well as larger portions of the genetic material including chromosomes. Accumulation of such genetic alterations can lead to the development of premalignant lesions and subsequent invasive carcinoma [1]. As depicted in Figure 1, there are modifiable risk factors which include smoking, tobacco, alcohol, diet/nutrition, lifestyle; while non-modifiable factors include age, ethnicity, socio-economic status, hereditary/family history. Tobacco is the major culprit and is associated with various cancers which is among the modifiable risk factor. Lack of awareness, delay in diagnosis and patients delay are attributed to the high incidence of oral cancer despite physical examination of oral cavity [2]. The 5 year survival rate is still at 50% of oral cancer patients [3]. The World Health Organisation highlighted India as a global epicentre of oral cancer and pointed out oral cancer as a major health challenges and burden.

According to population based cancer registry (rural and urban) of Ahmedabad (National cancer registry programme, Indian council of medical research), at The Gujarat Cancer & Research Institute, Ahmedabad, tobacco related cancers accounted for 55.2% of all cancers in males and 17.9% of all cancers in females. Among the tobacco related cancer sites in males, cancer of oral cavity was the most common site (29.7%) followed by cancer of tongue (20.5%). In females, cancer of esophagus alone accounted for (24.78%) of the total tobacco related cancers followed by oral cavity (19.8%) and tongue (17.3%). Thus, oral cavity is the predominant site among all the tobacco related cancers. Tobacco, betel quid chewing, tobacco, bidi and cigarette smoking and alcohol drinking are documented as major risk factors of oral cancer in India [4]. It has been reported that smokeless tobacco consumption is highly prevalent in Indian populations as compared to tobacco smoking and alcohol consumption [5].

Figure 1: Non-modifiable and modifiable risk factors of oral cancer.

Tobacco is used in smoking as well as smokeless forms. Though, cigarette smoking is seen in all Asian countries, bidi smoking is common in countries like India. Smokeless tobacco is used in a variety of forms and it has wide
spread among both men and women. Most commonly used forms are panmasala and gutkha. Panmasala and gutkha are blends of tobacco, areca nut, lime and catechu. This combination of ingredients is strongly genotoxic and carcinogenic [6].

According to global adult tobacco survey (2009-10), tobacco chewing is more prevalent than tobacco smoking. A greater incidence of oral cancer is observed in Asians where consumption of smokeless tobacco is high. This could be due to direct contact of tobacco and other carcinogenic compounds with oral mucosa for a longer time. Prevalence studies of tobacco use in India have shown wide variations between urban and rural areas, regions, age, gender, education, and other socio-demographic variables across the country. In Gujarat, tobacco chewing is the most frequent form of using tobacco than smoking by adolescents [7]. In addition, it is also documented that that every 4 out of 10 residents in Gujarat are found to be exposed to chewing tobacco with mawa-masala and gutkha [8]. Drinking alcohol is another important risk factor for cancer. Approximately 80 million people worldwide are affected by alcohol induced disorders [9]. Alcohol use may be under reported in regions like Gujarat where alcohol consumption is restricted. However, alcohol has a synergistic effect with tobacco chewing and smoking [10,11]. Acetaldehyde, the carcinogenic metabolite of alcohol, is responsible for oral carcinogeneiss [12]. Habituation or addictions to tobacco and alcohol use are in rise in younger generation [13]. Areca nut use is also reported to be an important risk factor for oral cancer. A considerable proportion of the world’s population is chewing areca nut and the habit is endemic throughout the Indian subcontinent. A large variety of ingredients, including tobacco, may be used along with areca nut constituting a betel quid [14].

There are certain key questions, which needs to be answered for tobacco related oral cancers:

- Is there any correlation of dietary factors with oral carcinogenesis process?
- Do antioxidant enzymes play a role in pathogenesis of oral cancer?
- Why all tobacco habituates does not get oral cancer? Who are more susceptible to oral cancer?
- Are there any differential changes in glycosylation observed between tobacco habituates and non-habituates?

To answer these questions, our Laboratory (Biochemistry Research Division, The Gujarat Cancer & Research Institute, Ahmedabad, Gujarat, India) is working on various aspects covering the above key questions. Our Laboratory is involved in studying several molecular signatures that could be distinctly correlated with biology of oral cancer. We have extensively explored multiple genes/pathways involved in tobacco metabolism, pathways involved in progression from normal to metaplastic/dysplastic and subsequently to cancer. The study included various aspects of oral cancer development.
Role of Sociodemographic and Dietary Factors in Oral Carcinogenesis

Studies contributing to the effects of diet were scanty reported from India, when our laboratory started working on this preventable cause of cancer. We conceptualized very early, way back in nineties that unraveling the effects of diet on cancer risk is of great public importance.

The study on vitamin B12 and folate status in oral cancer patients revealed a decrease in the plasma vitamin B12 and folate levels with respect to tobacco habits, disease progression, and vegetarian diet. The individuals in the lower quartile for vitamin B12 and folate were at a higher risk of developing oral precancerous conditions (OPC), as compared to those in higher quartiles. Similarly, the patients with OPC in lower quartiles were found to be at a higher risk of developing cancer than their counterparts. Folate levels were significantly lower in patients with advanced as compared to early disease. Our results documented potential significance of plasma vitamin B12 and folate levels in oral cancer [15]. Earlier studies have also observed protective role of folate against oral and pharyngeal carcinogenesis [16]. Studies have shown that oral epithelial dysplasia develop in person who expose to tobacco smoking and have low folate level [17]. Also high alcohol intake was associated with increased oral risk in women with low folate intake [18]. Earlier studies have depicted that high Vitamin B12 levels were associated with the risk of subsequently diagnosed cancer mostly within one year of follow-up [19]. Moreover studies have shown that optimal levels of micronutrients like Vitamin C, E, antioxidants, zinc, β-carotene and folate are effective in prevention of oral cancer [20].

Overall, our studies contributed that a high intake of fruits and vegetables reduces the risk of oral cancer. Our studies have also shown that plasma –carotene and vitamin-E levels were significantly lower in patients than the controls. The study clearly indicated that habit of tobacco consumption poor nutritional status and lack of awareness regarding association of diet with malignancy are major risk factors in the etiology of oral and pharyngeal cancer in Gujarat, India [21]. Thus, it can be suggested that socio-economic status and dietary pattern of individual is also one of the contributing factor for tobacco related oral cancer.

Role of Reactive Oxygen Species, Nicotine, Cotinine and Antioxidant Enzymes

Tobacco consumption leads to generation of free radicals, particularly oxygen radicals which play an important role in the complex course of multistep carcinogenesis (involved in both initiation and promotion of multistage carcinogenesis. Antioxidants scavenge free radicals directly, or interfere with the generation of free radical-mediated events, and inhibit the neoplastic process.
We performed a comprehensive study on the role of antioxidant enzymes on oral cancer etiopathogenesis. We have observed altered antioxidant enzymes (both Phase I and Phase II) in oral cancer patients. Erythrocytic superoxide dismutase (SOD) and plasma thiol levels were significantly lower, while glutathione peroxidase (GPx) and catalase (CAT) levels were significantly higher in controls with habit of tobacco (WHT) as compared to controls with no habit of tobacco (NHT). Glutathione S-transferase (GST), glutathione reductase (GR), SOD and CAT activities were significantly higher while GPx and thiol levels were significantly lower in oral cancer patients as compared to WHT controls. The data revealed that evaluation of antioxidant enzyme activities and thiol levels can be helpful to identify individuals at a higher risk of oral cancer development. Earlier studies have shown that SOD, catalase, GPx and non-enzymatic antioxidants Vitamin E, reduced glutathione (GSH) proceed synergistically with one another to detoxify the effects of lipid peroxidation. Increased levels of lipid peroxidation with decrease in SOD, catalase, reduced glutathione and GPx was observed in OSCC, which represents increased oxidative stress in oral cancer patients [22].

Our studies depicted that antioxidant enzymes were significantly higher whereas GPx and thiol levels were lower in patients as compared with habitual controls. Habitual controls with higher tobacco exposure and lower antioxidant enzymes as well as thiol showed higher risk of oral cancer development. Antioxidant enzymes were higher, whereas CAT and thiol levels were lower in malignant as compared with adjacent normal tissues. The study showed increased risk of oral cancer development in habitual controls with lower antioxidant enzymes, lower oxidative stress markers, and higher lifetime tobacco exposure [23,24]. Earlier reports have depicted that oral cancer patients had a significant decrease in salivary thiols as compared to controls, thus has role in pathophysiology of oral cancer [25].

The ROS and other reactive oxygen metabolites such as superoxide anions, hydrogen peroxide and nitric oxide are involved in multistep carcinogenesis process. Our data revealed that the alterations in antioxidant activities were associated with production of nitric oxide in oral cancer, which may have significant role in oral carcinogenesis process [26]. Earlier studies have observed a significant reduction in lipid peroxidation, SOD, catalase in the tissue of OSCC patients compared to controls. Moreover, reduced GSH and GPx were significantly increased in tumor samples, and a recent study suggested GSH as a potential prognostic marker [27].

In a separate study, we observed that urinary nicotine, cotinine and NO₂ + NO₃ levels were significantly elevated in WHT patients with OPC and oral cancer patients as compared with the NHT group. This was also the case of urinary thioether levels. Levels of urinary nicotine and co-
tinine were also higher in the non-abstinence group with oral cancers. The results confirmed that tobacco chewing and smoking habits are prominent risk factors for the development of oral cancer in the western part of India (Gujarat). Urinary nicotine, cotinine, NO₂ + NO₃, thiocysteine levels can be helpful for screening programs for oral cancer [28]. Earlier studies have also observed that nitrosative stress contributes to oral carcinogenesis [29]. Thus, reactive oxygen species, nicotine, cotinine, NO₂, NO₃ levels contribute to oral cancer development.

**Potentials of Tobacco Associated Genetic Susceptible Genes to Derive Risk Profiles in Oral Cancer**

Molecular epidemiological studies have provided concrete evidence that genetic predisposition along with tobacco consumption play an important role in the etiology of this malignancy. Measuring biomarkers associated with individual’s vulnerability to cancer due to tobacco exposure can be useful as early indicators of risk for preventive purposes and risk assessment.

Of particular interest are the enzymes involved in biotransformation and detoxification of tobacco-specific carcinogens and p53 gene which encode important tumor suppressor protein which plays a critical role in regulating cell cycle arrest, apoptosis and DNA repair. The polymorphisms in these genes have restrained effect on cancer risk at individual levels but may have a large population impact because the relevant polymorphism may be highly prevalent in the population. Ours is the first such epidemiological study to be carried out so far on the population of Gujarat from Western region of India. Our results revealed that the polymorphic variants of CYP1A1 gene did not show association towards oral cancer risk. The GSTM1 and GSTT1 null genotypes) were found to be over-represented in patients than controls, suggesting a moderate increase in risk of oral cancer. The oral cancer risk was significantly increased in the patients having either alone or concurrent deletion of GSTM1 and GSTT1. The results also proved significant association between tobacco habits, especially chewing, variant genotypes of CYP1A1, GSTM1 and GSTT1 and oral cancer risk. The data provided evidence that GST polymorphism modified the susceptibility to oral cancer and individuals with variant genotypes of the three genes with tobacco habits are at a significant risk of developing oral cancer [30]. Similarly CYP1A1 and GST polymorphisms have been known to play important role in head and neck cancer and can serve as cancer biomarkers [31].

The p53 polymorphisms especially Arg72Pro in exon 4 and its combination with 16 bp duplication in intron 3 and G>A transition in intron 6 could significantly modify the risk of oral cancer development in this population. Moreover, p53 codon 72 genotypes might be associated with tobacco associated oral cancer development [32]. Moreover, it was observed that frequency of p53 mutation was 52.2% in Gujarati population from our study [33].
The studies documented that the integration of these molecular signatures could be used to derive risk profiles of an individual.

**Differential Expression of Glycosylation Pattern in Tobacco Habituates and Non-Habituates and its Role in Oral Cancer Progression**

Our laboratory has the special research focus to define the biochemical changes in cell-surface glycoproteins that take place during all sequential stages to malignant transformation particularly in oral cancer (Figure 2). The odds ratio indicated a significantly higher risk for oral cancer among tobacco users and especially chewers. The levels of all the glycoprotein bands (192 kDa, 170 kDa, 116 kDa and 44 kDa) were higher in WHT patients than in NHT patients and were also higher in WHT controls than in NHT controls. Moreover, a 230 kDa glycoprotein consistently appeared only in individuals with tobacco habits and an increasing trend was observed from WHT controls to patients with OPC to WHT oral cancer patients. We termed this band as T-band (Tobacco band) and proved scientific evidences of ill-effects of tobacco in humans which is of utmost important to strengthen the fight against cancer. The results indicated potential utility of glycoprotein alterations in monitoring sequential changes occurring due to tobacco consumption during neoplastic transformation [34]. Further, mass spectrometry analysis can give insights into specific glycoproteins involved in tobacco related oral carcinogenesis. Recently there are newer trends which explore the opportunities and challenges related to glycomic and glycoproteomic analysis [35].

![Figure 2: Glycosylation: key regulator in oral carcinogenesis.](image_url)

**Evaluation of Tobacco Associated Salivary Biomarkers**

Though many studies have attempted to establish tumor markers in body fluids, still there are no definite biomarkers of oral cancer. Based on upcoming strong data on saliva as a diagnostic tool, saliva is receiving increased attention in recent years due to its various functions and
clinical utility. Proteins, mRNA, enzymes and chemicals extracted from saliva have been found at sufficiently distinct levels between OSCC and control samples to be considered as potential biomarkers [36]. Salivary biomarkers offer an easy, inexpensive, safe and non-invasive approach for disease detection [37,38]. Potential of salivary biomarkers to serve as a screening tool that does not rely on localization of a lesion allows them to identify patients with malignant and potentially malignant lesions [39]. Apart from its digestive and protective functions, there are various genomic, transcriptomic, proteomics and other metabolomic salivary biomarkers which have been studied in oral cancer as mentioned in Figure 3. These studies highlighted the potentials of salivary biomarkers in oral cancer screening, diagnosis, treatment monitoring and prognosis.

**Alterations in Tobacco Associated Glycosylation Changes in Saliva**

Sialylation and fucosylation; the major types of glycosylation changes are typical terminal modifications of proteins that mediate vital biological functions and also has implications in cancer [40,41]. Recently, it has been suggested that glycosylation is causally associated with all hallmarks capabilities [42]. Glycosylation thus has been suggested to be a key regulator in oral carcinogenesis (Figure 3). We observed significantly higher levels of serum and salivary TSA/TP ratio in patients with OPC and oral cancer patients as compared to the controls. Also, an increasing trend in serum and salivary α-L-fucosidase activity was observed from controls to patients with OPC to oral cancer patients, which was also supported by ROC curve analysis. The differential results observed in WHT vs. NHT were supportive of the premise that carcinogens such as tobacco are associated with molecular alterations observed in oral cancer progression. Moreover, the elevations in salivary levels of TSA/TP ratio and α-L-fucosidase activity as compared with serum, suggested the potential role of saliva in monitoring the changes associated with oral cancer development [43,44].

![Figure 3: Saliva: Its function and clinical utility of saliva-based biomarkers.](image-url)
Conclusion

The comprehensive review and our data suggest that an increasing knowledge of molecular genetic alterations, antioxidant status, role of dietary factors and markers associated in oral cancer progression has led to a better understanding of molecular pathways in the development of oral cancer. This new knowledge has put strong basis to generate new lead for prevention, early diagnosis and devising newer therapy for oral cancer. A robust progress has been made in our understanding of molecular basis of oral carcinoma, which will lead future research efforts to conduct scientific data. In addition, salivary based biomarkers can prove to an efficient non-invasive tool for its direct applicability to the clinics.

References


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