Review Of Literature
There is an increasing effort world-wide to determine the impact of environmental, genetic and lifestyle factors on genomic stability in human populations. As a result of rapid globalization and changing social attitudes, tobacco and betel quid chewing habits have been increasing worldwide. A number of diseases are associated with the use of tobacco since long and any approach aimed at expediting the detection of population at increased risk should be considered on a high priority task. Smokeless tobacco products are often made at home and are also manufactured commercially. Increasing habituation of the chewing product leads to the requirement of emergence of ready-to-use, easy to carry, non-perishable commercially available product. A cross sectional household sample survey conducted in 1998–99 covering 99% of Indian population in 26 states revealed that about 20% of the study population (28.1% men and 12.0% women) chewed tobacco/ pan masala (Rani et al., 2003). They reported that more than 50% men of Bihar, Arunachal Pradesh and Mizoram were indulged in the habit of tobacco chewing. On the other hand, above 30% women residing in West Bengal and Arunachal Pradesh consumed tobacco in chewing form while at Mizoram, the percentage reached to 60%. Areca, catechu and smokeless tobacco have been established to cause oral cancer (one of the ten leading cancers worldwide), irreversible gingival recession, oral submucous fibrosis, other oral pathologies, nicotine addiction and cardiovascular diseases, accidental inhalation (and its consequent complications) in children and worsening of asthma (Patel and Greydanus, 1999; Tariq, 1999; Merchant et al., 2000; Boucher and Mannan, 2002; Shah et al., 2002).
Areca nut is the seed of the oriental palm *Areca catechu* L. It has been estimated that, ~600 million people chew areca nut worldwide (Nelson and Heischober, 1999). It is one of the most profitable cash crops in India. Areca nut contains the closely related alkaloids arecoline, arecaidine, guvacoline and guvacine (IARC, 2004). Fig. 2 shows the areca nut alkaloids (Mujumdar *et al.*, 1982). Combined with the leaf of the betel vine *Piper betle* and slaked lime, various preparations of areca nut are prepared in India, Taiwan and Southeast Asia for the purpose of chewing. It has been estimated that areca nut (Fig. 3) chewing is the fourth most commonly abused drug, after the use of tobacco, alcohol and caffeine (Winstock, 2002). Several studies have reported a dependency syndrome associated with areca nut chewing (IARC, 2004; Winstock, 2002).

![Figure 2: Structures of the areca alkaloids found in areca nut](Adopted from Mujumdar *et al.*, 1982).

The major constituents of the nut are carbohydrates, fats, proteins, crude fiber, polyphenols (flavonols and tannins), alkaloids and mineral matter. The role of areca nut chewing in causation of oral cancer is established. Copper is implicated in tissues fibrogenesis via copper dependent enzyme (lysyl oxidase), which is crucial in stimulating fibroblasts in oral submucous fibrosis (Ma *et al.*, 1995). Thus, the copper content in samples of raw and processed areca nut were analyzed by Trivedy *et al.*, (1997). They found much higher copper content than that found most frequently in other nuts consumed by humans. The mean concentration of copper in samples of processed commercially available areca nut was found to be $18 \pm 8.7 \mu g/g$. 

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In an Indian Food Report, the copper content of processed areca nut was found to be 2.5 times that of the raw nut (Gopalan et al., 1991). The habit of chewing areca nut preparations without tobacco has been reported to be associated with an increased risk of oral cancer in four epidemiologic studies, one each from India, Pakistan, Taiwan and China (IARC, 2004). One prominent hypothesis put forwarded that various nitrosamines may be formed in the mouth from areca alkaloids and that these are causative of human oral cancer (Wenke et al., 1984 a,b,c). These nitrosamines include the nitrosamines of guvacine and guvacoline, together with 3-methylnitrosopropionitrile (IARC, 2004). An increased risk for the development of oral malignancy in “areca nut only users” is also reported (van Wyk et al., 1993; Merchant et al., 2000).

Areca nut is also causally linked to oral submucous fibrosis potentially malignant condition of the mouth, pharynx and oesophagus (Maher et al., 1994). Much effort has been expended in searching for the agents in areca nut which induce fibrosis. Recent studies suggest upregulation of the copper dependent extracellular enzyme lysyl oxidase by fibroblasts in oral submucous fibrosis is important, leading to excessive crosslinking and accumulation of collagen (Ma et al., 1995). IARC has evaluated betel quid without tobacco as causative agent of oral cancer and has mentioned that i) there is sufficient evidence in experimental animals for the carcinogenicity of areca nut, ii) there is limited evidence in experimental animals for the carcinogenicity of arecoline and iii) there is insufficient evidence in experimental animals for the carcinogenicity of arecaidine (IARC, 2004).

The usage of tobacco (Fig. 3) is practiced by some 1.1 billion people and up to 1/3 of the adult population worldwide (Gilman and Xun, 2004). The World Health Organization reported that it is to be the leading preventable cause of death worldwide and estimates that it causes 5.4 million deaths per year currently (WHO, 2008). Tobacco consumption is a major source of mortality and morbidity in India. According to estimates the expected number of death per year will reach 10 million by 2025 due to tobacco consumption.
Currently over 20% of worldwide tobacco related mortality occurs in India also (Gupta and Ball, 1990; WHO, 1999). Rates of smoking have leveled off or declined in developed countries, however they continue to rise in developing countries. A study on Gujarati migrant areca users in northwest London assessed their degree of dependency as equivalent to that of cocaine users especially if there is tobacco in the pan masala (Winstock et al., 2000).

Areca nut Tobacco

Figure 3: Chewing products (a) areca nut and (b) tobacco

To date, 28 carcinogens have been identified in smokeless tobacco (Brunnemann and Hoffmann, 1992). The major and most abundant groups of carcinogens are the non-volatile alkaloid-derived tobacco-specific N-nitrosamines (TSNA) and N-nitrosoamino acids. Because of the addictive properties of nicotine, tolerance and dependence develop. Also, 11 N-nitrosamino acids have been identified in smokeless tobacco: N-nitrososarcosine, N-nitrosoazetidine-4-carboxylic acid (NAzCA), 3-(methylnitrosamino)propionic acids (MNPA) and 4-(methylnitrosamino)butyric acids (MNBA), N-nitrosopropylene (NPRO), N-nitrosohydroxypropylene (NHPRO), N-nitrosopipeolic acid (NPIC), N-nitrosothiazolidine-4-carboxylic acid (NTCA), N-nitroso-2-methylthiazolidine-4-carboxylic acid (MNTCA), 4-(methylnitrosamino)- 4-(3-pyridyl)butyric acid (iso-NNAC) and 2-(methylnitrosamino)-3-phenylpropionic acid (MNPhPA) (Tricker and Preussmann, 1988; Hoffmann et al., 1995). All smokeless tobacco products
contain nicotine as a major constituent, which is addictive (Henningfield et al., 1997; Hatsukami and Severson, 1999). After nicotine is absorbed into the systemic circulation, it is rapidly distributed to all over the body. Once nicotine enters the blood, it is rapidly distributed to body tissue and plasma levels fall very quickly after intravenous administration. The distribution half-life of nicotine is estimated to be 9 min (Feyerabend et al., 1985). In humans, 85–90% of a dose of nicotine is converted metabolically before its excretion and only 5–10% is excreted unchanged in the urine. Nearly all nicotine metabolism occurs in the liver (Tricker, 2003). In the first stage, nicotine is largely converted to cotinine, the major hepatic metabolite of nicotine in humans. Cotinine is formed from the oxidation of nicotine at the 5 position of the pyrrolidine ring. Cotinine itself is further metabolized at a much slower rate than nicotine (plasma half life, 18 h) and only about 17% of cotinine is excreted unchanged in the urine (Benowitz et al., 1983). Major pathways of mammalian nicotine metabolism are shown in Fig. 4.

**Figure 4: Overview of major pathways of mammalian nicotine metabolism.**
(Adopted from Benowitz et al., 1994)
Despite strong campaigns against tobacco use through mass media and educational, legislative and other measures, the prevalence of tobacco use is still increasing in most of the developing countries. Hence in order to formulate and implement any control programme, apart from assessing the extent of the problem and identifying the risk factors, an understanding of the prevalent tobacco use behaviour, attitudes and beliefs among the community is also necessary (WHO, 1979).

Areca nut contains a number of psychoactive alkaloids, one of which is arecoline and affects the parasympathetic nervous system and also activates a sympathoadrenal response (Chu, 2002). Areca nut chewing has been shown to affect nervous system (Chu, 1995; Chu, 2001) as well as cardiovascular system (Bolinder et al., 1994; Chu, 2002; Lin et al., 2002). Peptic ulceration, reported to be increased in chewers of betel quid [with tobacco] (Ahmed et al., 1993). Chewers secrete more saliva on chemical stimulation, diluting salivary amylase and potassium and tobacco aggravates this effect (Reddy et al., 1980). It was hypothesized that serum levels of immunoglobulins may play an important role in the pathogenesis of oral mucosal diseases or reflect clinical changes in these conditions (Sisting et al., 2002). Shah et al., (1994) found that oral submucous fibrosis patients had higher serum levels of immunoglobulin IgG, IgM and IgA, whereas Canniff et al., (1986) found an increase in serum IgG levels in oral submucous fibrosis patients relative to normal individuals, but no difference in IgM and IgA levels was found. These findings suggest that nicotine is a potent immunopharmacological agent with regard to T-cell function (IARC, 2004).

**Oral health effects**

It is reasonable to believe that tobacco and areca nut chewing should have adverse effect on oral health. A growing body of evidence over the last five decades from epidemiological and experimental studies has shown that areca nut, even when consumed in the absence of tobacco or slaked lime, may have potentially harmful effects on the oral cavity (Trivedy et al., 1999).
Furthermore, it has been reported that use of betel quid and areca nut in any form is unsafe for oral health (Gupta and Ray, 2003).

**Effects on hard tissues**

The habitual chewing of areca nut may result in severe tooth wear involving incisal and occlusal tooth surfaces, particularly the enamel covering. The degree of attrition is dependent upon several factors, which include the consistency (hardness) of the areca nut, the frequency of chewing and the duration of the habit. Root fractures have also been demonstrated in chronic areca nut chewers, which are probably a consequence of the increased masticatory load and excessive and repetitive masticatory stress applied on teeth during chewing (Yeh, 1997; Gao et al., 2001).

It has been suggested that areca nut chewing may confer protection against dental caries. Epidemiological studies carried out in Southeast Asia suggest that the prevalence of dental caries in areca nut chewers is lower than that in non-chewers (Möller et al., 1977; Schamschula et al., 1977; Nigam and Srivastava, 1990). However, other data showed that there is no difference in the prevalence of dental caries between areca nut chewers and non-chewers in other Asian populations (Reichart and Gehring, 1984; Williams et al., 1996). Areca nut and tobacco reported to affect the oral hard tissue. The quid chewers have a higher prevalence of dental attrition and sensitivity than non-chewers. (Kumar et al., 2004; Parmar et al., 2008)

**Effects on soft tissues**

The study demonstrating a detrimental influence of betel chewing on the periodontal tissues was carried out by Mehta et al., (1955). They found higher prevalence of periodontal disease among betel chewers than non-chewers. Gupta (1964) found that the mean periodontal index (PI) for those who chewed betel nut was consistently greater than for those who did not chew. Waerhaug (1967) found that betel nut chewers over the age of 20 years had a very high PI indicating greater periodontal breakdown among chewers.
than non-chewers, even when subgroups of equivalent oral hygiene were compared.

A higher prevalence of gingivitis was reported among chewers of betel quid with tobacco (Amarasena et al., 2003). Ling et al., (2001) found that the levels of two periodontal pathogens, Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans, were higher in betel quid chewers who show a higher gingival index (an indicator of gingival inflammation) than in non-chewers. In addition, 77–87% of those who have gingival recession also have evidence of related oral mucosal pathology (WHO, 1988). In vitro studies by Chang et al., (1998) and Jeng et al., (1999) pointed towards the adverse effects of areca nut on the gingiva and periodontium.

Leucoplakia is the most common precancerous lesion associated with tobacco smoking and/or chewing. Tobacco smoking and chewing are the most important aetiological factors associated with leucoplakia. Leukoplakia is a clinical term used to describe patches of keratosis (Underwood, 2004). It is visible as adherent white patches on the mucous membranes of the oral cavity, including the tongue. It is sometimes described as precancerous (Ishida et al., 2007). Tobacco, either smoked or chewed, is considered to be the main culprit in its development (MFMER, 1998-2010). 5% to 25% of leukoplakias are premalignant lesions; therefore, all leukoplakias should be treated as premalignant lesions by clinicians - they require histologic evaluation or biopsy (Wikipedia). Those lesions remaining after two months cessation of smokeless tobacco should be considered to be true leukoplakias, possibly developed from or hidden by the previous smokeless tobacco keratosis. Leukoplakia is one of the commonest lesions in betel quid chewers. The WHO has classified these into two groups, homogeneous and non-homogeneous. Among non-homogeneous leukoplakias, nodular leukoplakia tends to show the highest rate of malignant transformation. Mehta et al., (1969; 1972) carried out house-to-house survey in various villages in India and reported that leukoplakia was usually associated with higher prevalence.
of chewing of betel quid with tobacco than chewing of betel quid alone or than no chewing habit.

Earlier Wahi et al., (1970) also showed that habit of chewing was associated with higher prevalence of leukoplakia than no chewing habit. No distinction was made between those chewing betel nut alone and those chewing betel nut with tobacco. Gupta (1984) reported a positive dose-response relationship between tobacco habit and the prevalence of leukoplakia. The most convincing evidence for the etiological role of tobacco came from intervention studies which demonstrated that leukoplakia regressed significantly more often when tobacco habits were discontinued or reduced compared to when the habits remained unchanged (Mehta et al., 1982). This is supported by other studies also (Mancini et al., 1965; Hirayama, 1966; Martin et al., 1999). Later, Kresty et al., (1996) reported an association between oral leukoplakia and two metabolites of the tobacco – specific nitrosoamine i.e. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). These two metabolites are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and [4-(methylnitrosamino)-1-(3-pyridyl) butyl]-beta-O-D-glucosiduronic acid (NNAL-Gluc). A significant association was found between the presence of leukoplakia and increasing levels of these metabolites demonstrating the role of smokeless tobacco in the causation of oral leukoplakia. Warnakulasuriya (1995) reviewed four case-control studies that examined relative risk of oral leukoplakia in betel quid chewers. In one of the studies, chewing areca nut (in betel quid without tobacco) raised the relative risk by at least three-fold compared with non-tobacco users. Further, the results of a case-control study conducted in Taiwan, where areca nut is chewed without tobacco also demonstrated that the cessation of areca nut chewing resulted in resolution of 62% of leukoplakias, suggesting that areca nut alone also has a significant etiological role in the development of leukoplakia.

Oral submucous fibrosis is a precancerous condition characterized by a progressive stiffening of the oral mucosa to the point wherein affected
persons have difficulty of opening their mouths. Oral submucous fibrosis (OSMF) is a chronic condition characterized by mucosal rigidity of varying intensity due to fibroelastic transformation of the juxta-epithelial layer (Murti et al., 1995). OSMF is a high-risk precancerous condition (Pindborg et al., 1984) with a malignant transformation rate of about 7.6% (Murti et al., 1985). Areca nut chewing could be one of the most important etiologic factors in OSMF (Sinor et al., 1990). One of the clinical symptoms of OSMF is trismus, a limitation of mouth opening. This may eventually impair the ability to eat and speak, and dental care may become difficult. Chewing betel quid has been recognized as one of the most important risk factors for OSMF (Kwan, 1976; Tang et al., 1997; Gupta et al., 1980; 1998; Sinor et al., 1990.), but only a fraction of betel quid chewers develops the disease. This disease affects 0.5% (5 million people) of the population in the Indian subcontinent and is a public health issue in many parts of the world, including the United Kingdom (Canniff et al., 1986), South Africa (Seedat and van Wyk, 1988) and many Southeastern Asian countries (Maher et al., 1994, Zain et al., 1997).

Lee and Chin (1970) observed the effects of betel nut chewing on the buccal mucosa of Indians and Malays in West Malaysia. They found varying degrees of epithelial atrophy, reduction of rete pegs, subepithelial inflammatory edema and fibrosis among chewers. A close association between OSMF and habituation to pan supari chewing was also reported by Dockrat and Shear (1970). Smith et al., (1975) found 0.49% prevalence of oral submucous fibrosis among the industrial workers in Gujarat, India. They observed that OSMF is more commonly associated with chewing of plain supari, or with combination of pan with chuna (slaked lime), kattha (paste of catechu), supari (betel nut) and tobacco than with other combinations. Gupta et al., (1980) demonstrated that the incidence of submucous fibrosis was nil among people with no chewing habit, whereas the incidence rate among the people who chewed areca nut along with or as an ingredient of pan was 35 per 1,00,000 people per year. Canniff et al., (1986) also analyzed forty-four patients with oral submucous fibrosis. All were areca nut chewers, of which some of them chewed along with additives as pan supari. Out of these, 68%
were heavy chewers while 37% were moderate chewers. Bhonsle et al., (1987) observed a variation in the pattern of occurrence of OSMF. In Pune, Maharashtra (India) OSMF affected more on the soft palate, uvula and retromolar region in contrast to patients in Ernakulam, Kerala (India) where the tongue, floor of the mouth and hard palate were more often involved. In addition, associated leukoplakia, oral cancer and petechiae were also observed in these groups of patients. The patients of Ernakulam, Kerala would spit out the juice macerated quid after chewing while the other group chewed for more time before swallowing the whole mixture. This could be the reason for the difference in sites of occurrence. A study was carried out among areca nut chewers in Taiwan showing that betel quid chewing is the main cause of OSMF and oral cancer (Eipe, 2005).

Babu et al., (1996) reported that habitual chewing of pan masala or gutkha is associated with earlier presentation of OSMF than betel quid use. Factors which might be responsible for these differences, are the tobacco content, the absence of betel leaf and its carotenes and the much higher dry weight of pan masala or gutkha as compared to betel quid i.e. pan. Gupta et al., (1997) selected a cohort of 12,212 villagers on the basis of their reported tobacco use in Ernakulam district, Kerala. They followed annually over a period of 10 years for education on tobacco habits and the development of oral precancerous changes in house-to-house surveys. Analysis of incidence rates revealed that some lesions were almost solely associated with smoking habits, whereas oral submucous fibrosis and oral lichen planus-like lesions were solely associated with betel quid chewing habits. Oral lichen planus and leukoplakia were associated with smoking as well as betel quid chewing habits. Gupta et al., (1998) undertook a study to determine whether there was an increase in the incidence of OSMF in the Bhavnagar district, Gujarat, India. The reported prevalence of OSMF in Bhavnagar district during 1967 was 0.16%. Among 5018 men who reported the use of tobacco or areca nut, 164 were diagnosed as suffering from OSMF. Areca nut was used mostly in the form of mawa, a mixture of tobacco, lime and areca nut and 10.9% of mawa users had OSMF. The disease as well as areca nut use was about 85%
among lower (< 35 year) age group and concluded an increase in the prevalence of OSMF, especially in the lower age groups, directly attributable to the use of areca nut products. Meghji et al., (1982) investigated the effects of areca nut on the inhibition of collagenase and thereby aid in the deposition of excess collagen. They observed that areca nut extracts, purified tannins and catechin (the diphenol tannin precursor), all inhibited collagen lysis by both bacterial and mammalian collagenases in a dose-dependant manner. They concluded that tannin from the chewed areca nuts might enhance the development of fibrosis in oral submucous fibrosis by reducing the susceptibility to collagen degradation by collagenase. Harvey et al., (1986) suggested that the unnatural accumulation of collagen in the tissue of OSMF patient is due to fibroblast proliferation and stimulation of collagen synthesis by the alkaloids in the areca nut as well as an inhibition of collagen degradation by the tannins and flavonoids which are also contained in the nuts.

The most serious aspect of OSMF is its high potential for development of cancer; the relative risk being 400 times (Gupta, 1999). The possible precancerous nature of OSMF was first described by Paymaster (1956) who observed occurrence of squamous cell carcinoma in one-third of patients with OSMF. Subsequent studies reported that the incidence of carcinoma varies in OSMF from 2 to 30% (McGurk and Craig, 1984). Awang (1986) investigated the pharmacology of betel nut (Areca catechu) in relation to OSMF and found that boiling the nut, commonly used for softening before chewing removed the majority of alkaloids. The variations in nut alkaloids and tannin content were probably due to plant variability and different procedures for the preparation of the areca nut for consumption. These variations in the pharmacologically active constituents of the betel nut may contribute to the regional difference in the incidence of the disease. It has been established that betel nuts (with or without paste of crude slaked lime and spices and/or tobacco) might play a role in the initiation and pathogenesis of OSMF. However, only a fraction of the betel nut chewers develop OSMF suggesting genetic susceptibility or lack of antifibrotic activity in OSMF cases. Canniff et
al., (1985) demonstrated a genetic predisposition to the disease, involving raised frequencies of HLA antigens A10, DR3. The histopathological and clinical features of the oral mucosa gave the suggestion of an autoimmune basis for OSMF. The results supported that OSMF is a chronic autoimmune disease, initiated by constituents of betel nut and occurring in genetically susceptible individuals.

Oral lichen planus is an inflammatory condition that affects oral mucosa. Oral lichen planus appear as white, lacy patches; red, swollen tissues or open sores. It may be important for malignant transformation, although its nature remains unclear (Murti et al., 1986). It has been categorized as a “probable precancerous condition” (Mehta and Hamner, 1993).

**Cancer of the oral cavity**

Oral cancer is currently the sixth most common malignancy in the world (Parkin et al., 2001). In India it is the most common type of malignancy among men and one of the five most common malignancies among women (Boyle et al., 1990). The etiology of oral squamous cell carcinoma (OSCC) is multifactorial, but tobacco usage in various forms continues to be an important risk factor (Ramesh et al., 1999). The frequency of oral cancer around the world is often indicative of the use of tobacco products (Parkin et al., 2005). South Asian communities are generally not aware that areca nut chewing can cause oral cancer and that ceasing its use would reduce the likelihood of developing oral cancer (Vora et al., 2000; Shetty and Johnson, 1999). Squamous cell carcinoma is quite naturally the most worrisome mucosal change encountered in smokers and tobacco’s role in producing squamous carcinomas of the oral, pharyngeal and laryngeal mucosa is well established. Fifty eight percent of the 390,000 oral and oro-pharyngeal cancers of the world occur annually in South and Southeast Asia (WHO, 2003). A study in the UK showed that many Bangladeshi adolescents living in East London were unaware of the association between areca nut chewing and oral cancer (Prabhu et al., 2001). Reports also suggest that many shopkeepers selling
these chewing products are not aware of any health risks and there are no restrictions placed on sale of these products to minors (Warnakulasuriya, 2000). Further, it has been established that there is a dose-response relationship between the amount of tobacco product used and the development of oral cancer (Parkin et al., 2005; IARC, 2004).

The mouth is the only body site that permits viewing with the naked eye the ravages of smoked and smokeless tobacco. It is often possible to view in the mouth during a clinical examination normal tissue, premalignant lesions (e.g., leukoplakia) and malignant tumors (Taybos, 2003; Silverman, 2003). Smokeless tobacco use has been implicated for the etiology of the oral pre-cancerous and cancerous lesions. In the South Asian region over one-third of tobacco consumed is smokeless (Gupta and Ray, 2003). Subjects with confirmed oral precancers are advised to quit using tobacco and encouraged to undergo removal of excisable non-homogeneous oral leukoplakias, if present (Gupta et al., 1986). Oral cancer occurs more commonly among men than women depending upon the extent and type of tobacco habits prevalent. Betel quid chewing is the major risk factor for buccal mucosal and gingival cancer. Betel quid contain both carcinogens and genotoxic agents which have role in multistage progression of oral cancer (Jeng et al., 2001). Smokeless tobacco contains nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; areca nut contains arecoline and 3-(methylnitrosamino) propionitrile, while lime provides reactive oxygen radicals, each of which has a role in oral carcinogenesis (Nair et al., 2004). Chewing betel quid without tobacco is an independent risk factor for developing oral cancer (Jacob et al., 2004). When betel quid with tobacco is consumed with alcohol and smoking the relative risk increases by 11-fold (Subapriya et al., 2007).

Cross-sectional studies conducted by Mehta et al., (1969; 1972) provide the prevalence of oral cancer among people with or without tobacco habits. In these studies over 1,50,000 individuals, although there were a substantial number of non-habituates, 14 of 38 oral cancers occurred in people who were solely betel-tobacco chewers, 24 among other tobacco
chewers and none among non-chewers. In the most extensive study of its kind, Gupta et al., (1980) followed up 30,000 individuals over a 10-year period in three areas of India. The betel-tobacco chewing habit was common in Ernakulam, Kerala and the annual age adjusted incidence of oral cancer was 23 per 100,000 among betel-tobacco chewers. In another study the incidence of oral cancer among persons with smoking and pan supari chewing habit was 0.09% as against 0.02% among non-users (Smith et al., 1975). A study of Navajo students found that the prevalence of oral lesions tends to increase with duration and frequency (days per week) of use of oral tobacco (Wolfe and Carlos, 1987). Also, they found minimum significant increase in the presence of oral lesions among oral tobacco users that were associated with the concomitant use of smoking.

The survival curves of oral cancer have plateaued over the past two decades and remain among the worst of all cancer sites. It is now clear that we need to advance our understanding of oral cancer etiology and development before further improvement can occur (Schantz, 1993).

**Cytogenetic Alterations**

Cytogenetics is a branch of genetics, that is concerned with the cells especially the study of the structure and function of chromosomes. The cytogeneticists could easily identify chromosomal defects, subtle deletions, inversions, insertions, translocations, fragile sites and other more complex rearrangements and refine break points (Britto and Ravindran, 2007). Cytogenetic markers such as chromosomal aberrations (abnormality in chromosomal structure and number), micronuclei frequency is relatively rapid, facile and sensitive indicators of genetic damage, which are useful for the diagnosis of genetic disorders. Abnormalities in chromosomal structures such as increased chromosomal breakage or chromosomal loss are associated with enhanced risk of carcinogenesis and in the progression of neoplastic transformation (Poppe et al., 2007). Genetic damage at the chromosomal level entails an alteration in either chromosome number or chromosome structure and such alterations can be measured as CA or MN frequency.
Conventional techniques for measuring chromosomal abnormalities require proliferating cells so that chromosomes can be seen at mitosis. Micronuclei are acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division and appear in the cytoplasm of interphase cells as small additional nuclei. In contrast to the CA evaluation the scoring of micronuclei in lymphocytes is simple and fast. Increased levels of CA have been associated with increased cancer risk (Hagmar et al., 1994; 1998). However, high levels of SCE and MN frequency have been observed in persons at higher cancer risk due to occupational or environmental exposure to a wide variety of carcinogens (Fenech et al., 1997; Vaglenov et al., 1999; Somorovska et al., 1999; Fucic et al., 2000). The use of exfoliated cells for MN assays has become well established in epidemiological studies aimed at defining genotoxic effects on target tissue following chronic exposure to epithelial carcinogens (Smith et al., 1993).

A key-initiating step in the carcinogenic process is the formation of DNA adducts. Some miscoding DNA adducts that could be formed by use of chewing tobacco. Persistence of these adducts during DNA replication can cause miscoding, leading to mutations and derangement of cellular growth control processes. The tobacco-specific nitrosamines can induce miscoding DNA adducts, including $O_6$-pyridyloxobutyl and $O_6$-MeG adducts (Hecht, 2003), that could initiate the tumourigenic process in the oral cavity and leading to focal areas that progress at different rates towards invasive cancer (Califano et al., 1996; Oh and Mao, 1997). DNA extracted from exfoliated oral mucosal cells collected from Canadian non-smoking controls and Indian areca nut chewers were used for analysis of aromatic DNA adducts by the $^{32}$P-postlabelling technique (Dunn and Stich, 1986). Differential amounts of five aromatic DNA adducts were found within these groups, but there were no differences among the groups (tobacco chewing, betel quid chewing or smoking). Chen et al., (1999) identified several safrole-like DNA adducts from 77% of tissues of oral squamous-cell carcinomas in Taiwan, China. Six of seven oral submucous fibrosis tissues obtained from betel quid chewers also exhibited the same safrole-like DNA adducts. In many studies the levels of
carcinogen-DNA adducts have been shown to be higher in tissues of smokers than in tissues of non-smokers (Nakayama et al., 1984).

Cultured human oral epithelial carcinoma cells produced reactive oxygen species following in vitro incubation with an aqueous extract of smokeless tobacco (Bagchi et al., 1996). Smokeless tobacco extracts significantly induced the production of superoxide anion and increased lipid peroxidation, DNA fragmentation and protein kinase C activity in primary cultures of human oral keratinocytes (Bagchi et al., 1997; 2002). Using flow cytometry with the fluorescent dye, propidium iodide, a dose-dependent increase in apoptotic cell death was observed following treatment with smokeless tobacco extract which was inhibited by several antioxidants including vitamin C and vitamin E (Bagchi et al., 1999). Smokeless tobacco extract that contained an equivalent amount of nicotine was found to be more toxic than nicotine in the generation of reactive oxygen species, as assessed by the measurement of changes in GSH and malondialdehyde levels in Chinese hamster ovary cells (Yildiz et al., 1999). The situation is aggravated by further induction of phase I enzymes and suppression of antioxidant systems, such as the GSH/GST system. Reduced plasma levels of several antioxidant vitamins have also been reported in smokeless tobacco users (Yildiz et al., 1999). Areca nut extract (ANE) produces adverse effects on the proliferation of phytohaemagglutinin-stimulated human lymphocytes in vitro, suggesting that there may be impaired immune surveillance in areca nut chewers (Yang et al., 1979). A dose-dependent induction of apoptosis mediated by nitric oxide was observed in HCPC-1 cells treated with smokeless tobacco extracts (Mangipudy and Vishwanatha, 1999). Fox et al., (1995) demonstrated that cell death following long-term snuff exposure of human fibrosarcoma (HT-1080) cells in vitro is not a result of apoptosis but is related to epithelial–mesenchymal interactions that result in the loss of cell adhesion.

**Biomarker Study**

The use of a biomarker as an indicator of disease development is that the marker will translate into a relationship between exposure and disease
(Schatzkin et al., 1990). The biomarkers of exposure and effect and clinical disease can all largely be influenced by host susceptibility factors, which include polymorphisms that alter the activity of relevant DNA repair, carcinogen metabolism and apoptotic pathway genes, as well as dietary factors that alter the activity of such genes (Fenech, 2002).

The buccal mucosa provides a barrier to potential carcinogens that can be metabolized to generate potential reactive products (Vondracek et al., 2001; Spivack et al., 2004). As up to 90% of all cancers appear to be epithelial in origin, the buccal mucosa could be used to monitor early genotoxic events as a result of potential carcinogens entering the body through ingestion or inhalation (Cairns, 1975; Rosin, 1992; Holland et al., 2008). Exfoliated buccal mucosa cells have been used non-invasively to successfully show the genotoxic effects of lifestyle factors such as tobacco smoking, chewing of betel nuts and/or quids, medical treatments, such as radiotherapy as well as occupational exposure to potentially mutagenic and/or carcinogenic chemicals and for studies of chemoprevention of cancer (Stich et al., 1982a; 1988; Stich and Rosin, 1983; Holland et al., 2008). With regard to exposure to radiation it has been shown by Moore et al. (1996) that the buccal micronucleus cytome can detect a 16-fold increase in micronucleus (MN) frequency in oral cancer patients after completion of treatment of photons. The buccal micronucleus cytome has been used to measure biomarkers of DNA damage (micronuclei and/or nuclear buds), cytokinetic defects (binucleated cells) and proliferative potential (basal cell frequency) and/or cell death (condensed chromatin, karyorrhexis, pyknotic and karyolitic cells).

In a multicountry study, Stich et al. (1986) scored scraped or brushed micronucleated epithelial cells from oral mucosa. The number of betel quids consumed was an average of 44 quids each day in Taiwan, China, compared with an average of 20 quids per day in India (Stich et al., 1982b). Betel quid chewers in Orissa (India) who regularly chewed dried areca nut, slaked lime, betel leaf, tobacco and catechu had the highest frequencies of micronucleated
cells, followed by those chewed fresh areca nut, slaked lime and betel leaf. Stich et al., (1991) explored the possibility of reversal of the formation of micronuclei in the oral cavity using vitamin A and/or β-carotene. The treatment decreased the frequency of micronuclei in users of betel quid with tobacco. However, the reappearance of micronucleated cells was noted after termination of the treatment (Stich et al., 1991). Stich et al., (1982 a, b) used the micronucleus assay to exfoliated oral epithelial cells of khaini tobacco chewers of Bihar, India also and reported an elevated frequency of MN in all the individuals examined.

Among areca nut specific nitrosamines (ASNA), 3-(methylNitrosoamino) propionaldehyde (MNPA) and their precursor alkaloids was the most potent inducer of DNA single strand breaks in human buccal epithelial cells in vitro (Sundqvist et al., 1991). Arecoline modulates the activity of matrix metalloproteinases and their tissue inhibitors and lysyl oxidase which leads to the accumulation of collagen in the oral buccal mucosal fibroblasts in vitro (IARC, 2004). Nair et al., (1990) collected slaked lime samples from Papua New Guinea, where the incidence of oral cancer was high among chewers. In 25 lime samples, the free Ca(OH)$_2$ and pH were highly correlated with the generation of ROS from areca nut extract in vitro and DNA damage in vitro measured as 8-OH-dG (8-hydroxy 2-deoxyguanosine). Fe$^{2+}$ and Mg$^{2+}$ levels in the lime samples were too low to modify the formation of ROS, but H$_2$O$_2$ formation was almost entirely inhibited by addition of Mg$^{2+}$ to the reaction mixture. These results suggest that the Ca(OH)$_2$ content of lime in the presence of areca nut is a major factor responsible for the formation of ROS which cause oxidative damage in the DNA of buccal mucosa cells of betel quid chewers (Nair et al., 2004).

Elevated levels of micronucleated cells were found in the oral mucosa of khaini chewers from Bihar, India (2.1%; 0.8-4.9%), gutkha chewers from Orissa, India (0.7%; 0.3-1.8%) and naswar users from Uzbekistan (4.1%; 2.7–5.7%) compared with controls (non-chewers, nonsmokers) from various locations (0.5%; 0.0–1.0) (Stich and Anders, 1989). Localized micronucleus
formation in the oral mucosa was described in *khaini* chewers; 2% of the cells in the gingival groove showed micronuclei (Stich *et al*., 1992). A study from India, 6.3% of the buccal mucosal cells were micronucleated in chewers of tobacco with lime (Ghose and Parida, 1995). In a study designed to monitor genotoxicity in the bidi industry that included tobacco processing plant workers and bidi rollers who did not use tobacco, the mean frequency of micronucleated cells in the buccal epithelium was significantly higher among bidi rollers and plant workers than among non-exposed controls (Bagwe and Bhisey, 1993).

Very recently, the MN and other nuclear anomalies reflect the genetic and cytotoxic damage, associated with tobacco and areca nut consumption reported by us (Joshi *et al*., 2011). Hande and Chaudhary (2010) observed progressive decrease in cellular diameter, increase in nuclear diameter and increase in ratio of nuclear diameter to cellular diameter in smears from all tobacco users as compared to normal subjects and indicated the cause-effect relationship between tobacco and quantitative alterations. Kausar *et al*., (2009) showed that sadagura, a unique smokeless tobacco preparation, consumed as a single agent or in combination with betel quid, leads to a significant induction of cytogenetic damage in the buccal epithelial cells of habituates.

Micronuclei are ideally scored in binucleated cells and can only be expressed in dividing eukaryotic cells (Fenech, 2000). They are mostly studied in cultured isolated lymphocytes stimulated to divide by phytohaemagglutinin. Cytochalasin-B is an inhibitor of actin polymerisation, preventing the formation of the microfilament ring that constricts the cytoplasm between two daughter nuclei during cytokinesis (Fenech, 2000). This action causes the accumulation of binucleated cells in almost all dividing cells. The use of Cytochalasin-B was introduced into the original micronuclei assay to enable reliable comparisons of chromosome damage between cells with different cell cycle kinetics (Fenech, 2000). Despite considerable variation in methods and variables influencing the measurement, such as
incubation periods, reagents and cell fixation, the induction of micronuclei is accepted as an effective biomarker of DNA damage. However, to validate the assay as a reliable biomarker of genotoxicity, the International Collaborative Project on Micronucleus frequency in Human Population (HUMN) was initiated to compare data on micronuclei frequency in different population and cell types (Fenech et al., 1999). As a result, a protocol has been developed and published (Kirsch-Volders et al., 2003) while Fenech et al., (2003) compiled detailed scoring criteria for the CBMN assay, using isolated lymphocytes cultures as reference. The CBMN assay is a genotoxicity assay, which can be used to detect a variety of chromosomal damage endpoints that reflect chromosomal breakage, chromosome rearrangements, and gene amplification.

Mutations in cells are known to play an important role in induction of cancer. Short-term assays like the micronucleus test (MN), chromosomal aberration (CA) and DNA damage by comet assay are sensitive and well-established cytogenetic markers of DNA damage. Dave et al., (1991) compared 30 healthy controls and 15 pan-masala consumers with respect to cytogenetic effects in peripheral blood lymphocytes and exfoliated cells from buccal mucosa. Sister chromatid exchange and chromosomal aberrations of peripheral blood lymphocytes were more frequent in cells of pan-masala chewers than in those isolated from control subjects. The frequencies of micronuclei in exfoliated mucosal cells were shown to be higher consistently in chewers of tobacco plus slaked lime, mawa, tamol and areca nut in different regions of India compared with healthy individuals with no habit (Kayal et al., 1993). They further reported no correlation between the frequencies of micronucleated cells and the duration or frequency of the chewing habit and also the frequencies of micronuclei were shown to be higher in healthy chewers and oral submucous fibrosis patients. Several studies have shown a relationship between snuff user's hyperkeratosis and elevated frequencies of micronucleated cells and/or chromosomal aberrations and sister chromatid exchange in snuff users compared with non-users (Livingston et al., 1990; Tolbert et al., 1991, 1992; Roberts, 1997). Swedish
moist snuff users showed increased mitotic rate, increased cell density and loss of cell cohesion (Larsson et al., 1991). A total of 44 subjects were examined by Sellappa et al., (2009a) and the mean percentage of MN was found to be 1.90 ± 1.03 in chewers, 2.00 ± 1.12 in chewers with smoking habits and 0.81 ± 0.66 in controls. It concludes that a mixture of betel leaf, areca nut and tobacco is unsafe for oral health.

Higher frequencies of micronucleated cells and/or chromosomal aberrations and/or sister chromatid exchange were also reported in smokeless tobacco consumers and in patients with oral squamous-cell carcinoma, in comparison to non-user controls (Stich et al., 1982a,b; Nair et al., 1991; Das and Dash, 1992; Kayal et al., 1993; Trivedi et al., 1995; Ozkul et al., 1997). Microsatellite analysis in squamous cell carcinoma of head and neck for allelic loss at 10 major chromosome loci demonstrated that the spectrum of chromosomal deletions progressively increases at each histopathological step from benign hyperplasia to dysplasia to carcinoma in situ to invasive cancer (Califano et al., 1996). The most common gains in tobacco chewing associated oral cancers are on chromosomes 8p, 9p, 9q, 11q, 17q and 20q and the most frequent losses are in chromosome arms 3p, 4q, 5q, 9q and 18q (Mahale and Saranath 2000; Lin et al., 2002; Pai et al., 2002).

It has been shown that there is a probable risk of oral carcinogenesis in healthy tobacco consumers having higher CA and life time chewing exposure and hence the deficient DNA repair capacity of oral cancer patients might be due to the disease process or the tobacco exposure (Patel et al., 2010). Chewing Khaini (only tobacco with lime) damages chromosomes, in the form of loss of heterozygosity, identified on the long arm of chromosome 2 (2q), the short arm of chromosome 3 (3p) and the long arm of chromosome 21 (21q) of oral cancer cases who had quit chewing habit from more than 10 years duration and had chewed 10-15 times a day (Choudhury et al., 2009). Sellappa et al., (2009b) found significant differences in the micronucleus (MN), Comet scores and chromosomal aberrations (CA) between smokeless
tobacco users and control subjects. These findings provide evidence for the view that polymorphisms in DNA repair genes may modify individual susceptibility to tobacco related cancers and justify additional studies to investigate their potential role in the development of cancer. One of studies shows that the oral use of smokeless tobacco represents a genotoxic hazard, which is even higher than the DNA damage by comet assay observed in cigarette smokers (Sardas et al., 2009). Earlier, the comet assay has repeatedly been used to measure DNA damage related to tobacco smoking. Some studies were performed specifically to investigate a potential effect of smoking using the comet assay, whereas various other studies determined the effect of smoking as a potential confounding factor in the course of occupational studies (Moller et al., 2000; Faust et al., 2004).

Aqueous and dimethyl sulfoxide (DMSO) extracts of both pan masala plain and gutkha induced CA, SCE and MN in CHO cells in the presence and absence of an exogenous metabolic system, although metabolic activation markedly inhibited the chromosomal damaging effect, implicating the presence of direct-acting mutagens and clastogens (Jaju et al., 1992; Patel et al., 1994a). They suggested that the decrease in genotoxic response in the presence of an exogenous metabolic system is probably due to detoxification of mutagens by microsomal enzymes. The clastogenic effect of pan masala extract towards CHO cells was further evaluated in the presence of ethanol, which showed a marked decline in the mitotic index and elevation in the frequency of CA (Patel et al., 1994b). ANE extract exposure also inhibited the growth of CHO cells in a dose- and time-dependent manner (Lin et al., 2009). They further reported an increase in MN frequency, G2/M arrest, cytokinesis failure and an accumulation of hyperploid/aneuploid cells and suggested an increase in intracellular H₂O₂ level and actin filament disorganization as a possible mechanism. ANE comprising of 30-100 kDa fractions which is mainly composed of carbohydrates and to a lesser extent, of proteins, are capable of triggering autophagy in carcinoma cell lines (Lin et al., 2008).
Genomic instability

Analysis of cytogenetic changes in betel quid and tobacco associated oral squamous cell carcinomas showed most common gains in chromosomes 8q, 9q, 11q, 17q and 20q and most frequent losses in chromosome arms 3p, 4q, 5q, 9p21–23 and 18q, a high frequency of breakage and exchanges at the 1cen-1q12 region and allelic imbalance in short tandem repeat markers (Rupa and Eastmond, 1997; Mahale and Saranath, 2000; Lin et al., 2002; Pai et al., 2002). Loss of 3p was significantly associated with poor survival of patients (Lin et al., 2002). Lee et al., (2001) showed mitochondrial DNA (mtDNA) deletions (4977-bp deletion) in oral squamous-cell carcinomas of betel quid chewers. They further revealed that the mtDNA deletions detected in oral tumours were less abundant than those in the surrounding non-tumorous tissues. Moreover, betel quid chewing also shown to significantly enhanced the accumulation of mtDNA deletions in non-tumorous oral tissues.

The p53 gene is one of the most commonly mutated genes in all types of human cancer. Analysis of somatic tissue from many human cancers has shown that the wild-type p53 allele is frequently lost and a mutant allele retained, providing a growth advantage for malignant cells (Vogelstein and Kinzler, 1992; Harris, 1993; Jensen and Page, 1993). The mutation of the p53 gene can damage its DNA-binding properties and impair cell-cycle control and cell proliferation (Roy et al., 1994). Of 14 polymorphisms in the p53 gene, three have been widely studied, including a G to C polymorphism at codon 72 (proline/arginine), a 16bp insertion (in intron 3) and a G to A transition (in intron 6). The association between p53 polymorphisms and tobacco-related cancer risk is highlighted in several lung cancer studies. In a case–control study with 635 pairs of lung cancer patients and healthy controls, Wu et al., (2002) found that variant alleles of p53 exon 4, intron 3 and intron 6 were associated with increased lung cancer risk. Similarly, the variant haplotypes were also associated with an increased risk for lung cancer. Based on meta-analysis of p53 polymorphisms and lung cancer risk, by including 16 case–control studies, Matakidou et al., (2003) concluded that individuals with p53 exon 4 Pro/Pro genotypes had a 1.18-fold increased lung cancer risk (95% CI:
Association between TP53 mutations in oral carcinomas with betel quid chewing is limited. Studies demonstrated infrequent TP53 mutations in oral cancers in Southeast Asia including Sri Lanka, Taiwan, China and India, as well as in Papua New Guinea (Chiba et al., 1998; Hsieh et al., 2001). No TP53 mutations were observed in oral leukoplakia or squamous-cell carcinomas of chewers of betel quid without tobacco in Indian populations (Heinzel et al., 1996; Ralhan et al., 2001), or in 48 cases of oral tumour from eastern India in chewers of betel quid with tobacco (Patnaik et al., 1999). However, in Sri Lankan populations, Chiba et al. (1998) reported TP53 mutations in 10/23 (43%) oral squamous-cell carcinomas in betel quid chewers. In addition to point mutations in G + C-rich regions, small deletions or insertions were also observed (Chiba et al., 1998). The G:C→C:G transversions were observed in betel quid chewers or smokers and codons 135 and 136 were frequently mutated (G→T; A→G) in oral squamous-cell carcinomas associated with betel quid and tobacco consumption (Chiba et al., 1998; Hsieh et al., 2001; Ralhan et al., 2001). Several studies reported a high incidence of p53 protein expression in oral premalignant lesions and squamous-cell carcinomas from betel quid and/or tobacco consumers. All cancers were detected among betel quid chewers who included tobacco in their quid and/or smoked. However, the acquisition of p53 protein expression in 9/10 biopsies that did not show p53 expression at baseline occurred once they had undergone progression to squamous-cell carcinoma. Trivedy et al. (1998) reported p53 protein expression in 15/20 (75%) oral submucous fibrosis cases, 3/6 (50%) squamous-cell carcinomas arising from oral submucous fibrosis and 15/21 (67%) squamous-cell carcinomas not arising from this disease. Moreover, the combination of telomere dysfunction and p53 deficiency has been shown to accelerate tumorigenesis in vivo (Chin et al., 1999; Gisselsson et al., 2001). Although telomerase is reactivated in most
human cancers, telomere shortening and dysfunction might impair chromosomal stability early in carcinogenesis and, consequently, drive the initial carcinogenic process.

In a study of toombak (Sudanese snuff) users, four head and neck squamous-cell carcinomas from three patients who used toombak and one patient who did not use toombak were screened for TP53 mutations (Lazarus et al., 1996a,b). Mutations were found in tumours resected from two of three toombak users, one at codon 282 (C→T) and the other in intron 6 (AT→GC). No K-RAS (codons 12 and 13) or H-RAS (codon 12) mutations were found in tumours that harboured TP53 mutations and the other tumours. A high incidence of H-RAS mutations (codons 12, 13 or 61) was reported in oral cancers from India, the majority of which were in tobacco chewers (Saranath et al., 1991). Xu et al., (1998) analysed four oral squamous-cell carcinomas from snuff users or chewing tobacco and 16 oral squamous-cell carcinomas from smokers only. Two of the tumours from users of snuff or chewing tobacco showed TP53 mutations, while p53 protein accumulation was observed in all four tumours. No differences were observed in the p53, cyclin D1 and Rb profiles of users of smokeless tobacco and cigarette smokers. TP53 mutations in 56 oral squamous-cell carcinomas from Sudanese toombak dippers and non-dippers and from Scandinavian non-dippers were analysed by Ibrahim et al., (1999). No TP53 mutations were found in non-malignant oral lesions from toombak dippers or non-dippers from Sudan. TP53 mutations in exons 5–9 were found in 13/14 (93%) toombak dippers compared with 8/14 (57%) non-dippers from Sudan and 17/28 (61%) non-dippers from Scandinavia. Mutations G:C→A:T; C:G→T:A; G:C→T:A which are known to be associated with TSNA were found to be most common in oral squamous-cell carcinomas from toombak dippers which suggests a possible role of TSNA in the induction of TP53 mutations in these tumours. Saranath et al., (1999) reported TP53 mutations in 14/83 (17%) oral squamous-cell carcinoma patients from India, the majority of whom were tobacco chewers.
Role of copper and zinc

Majority of the studies available point towards the role of areca nut in causation of OSMF. However, only a fraction of the betel nut chewers develop OSMF suggesting genetic susceptibility or lack of anti-fibrotic mechanism. In addition to areca nut and tobacco chewing, potential role of copper in causation of OSMF could also be considered as areca nut contains appreciable amount of copper and soluble copper release in the mouth while chewing areca nut. Copper is implicated in tissues fibrogenesis via copper dependent enzyme i.e. lysyl oxidase, which has a crucial role in cross-linking of collagen & elastin. Lysyl oxidase has been also implicated in other fibrotic diseases i.e. Hepatic, Pulmonary fibrosis & Scleroderma. Copper is an essential co-factor required for the expression of Lysyl oxidase (Harris et al., 1980). Recent evidence suggests upregulation of the copper dependent extracellular enzyme lysyl oxidase by fibroblasts in submucous fibrosis is important, leading to excessive cross-linking and accumulation of collagen (Ma et al., 1995). They suggested that there is an up-regulation of the copper dependent extra-cellular enzyme lysyl oxidase, which stimulates fibroblasts in oral submucous fibrosis, leading to excessive cross linkage and accumulation of collagen. Copper is an essential trace element for the function of several key enzymes involved in human metabolism (Linder and Hazegh, 1996). These include cytochrome-c oxidase, superoxide dismutase, metallothionein and lysyl oxidase. Abnormalities in copper absorption, metabolism and excretion can lead to deposition of copper in several body sites. These include genetic disorders like Wilson’s disease (Taylor, 1996) or environmental contamination leading to copper accumulation in Indian childhood cirrhosis and pulmonary fibrosis (Baker et al., 1995). Earlier Trivedy et al., (1997) demonstrated that areca products contain a high level of copper (mean 302 nmol/g) when compared to other commonly eaten nuts (22-173 nmol/g) and that soluble copper is released into whole mouth saliva following chewing areca for 5-30 minutes. Owing to these, copper might also have a significant role in the development of OSMF, as there is a report that supports the hypothesis of copper as a factor in OSMF (Trivedy et al., 1999).
Zinc deficiency has been associated with adverse health effects in humans and animals, however, over exposures to zinc also have been associated with toxic effects. (ATSDR, 1994). The regeneration of the oral mucosa is zinc and vitamin A dependant. Hyperkeratosis, a disturbed immune system and a high incidence of oral malignancies have been reported in cases of zinc deficiency by Kleier et al., (1998). They observed significant lower level of zinc in leukoplakia and oral cancer patients compared with the control group. Jayadeep et al., (1997) also reported a significant decreased zinc level only in male patients with leukoplakia and squamous cell carcinoma. However, they noted the significantly increased level of copper in oral leukoplakia and cancer patients in both sexes. But, earlier Varghese et al., (1987) noted a significant reduction in the serum copper and zinc levels in both OSMF and oral cancer. Yoshida (1989) studied the effects of zinc deficiency on rats’ oral mucosa and observed hyperkeratosis in buccal mucosa, ventral surface and interpapillary mucosa of the tongue in rats fed with zinc low diet. He also suggested that zinc deficiency might have a serious consequence on the oral mucosa in its tolerance.

In addition, measurement of cotinine in biological fluid is useful as a tobacco exposure biomarker. Cotinine is the major metabolite of nicotine and is the analyte of choice as it fulfils the prerequisites of specificity and retention time (18–20 h) and is found at detectable levels in all the matrices (Idle, 1990; Domino, 1995). It can be used for tobacco exposure quantification in both actively and passively exposed individuals (Benowitz et al., 1983; Zevin et al., 2000). Cotinine, however, is biotransformed into secondary metabolites such as cotinine glucuronide, 3-hydroxy-cotinine and 3-hydroxy-continine glucuronide (Benowitz et al., 1983; DeLeon et al., 2002). Plasma cotinine concentrations correlate better to various measures of biologic effects of cigarette smoking than self-reported cigarettes per day does (Perez-Stable et al., 1995; Benowitz and Sharp, 1989). Cotinine concentrations in plasma, urine and saliva of nonsmokers have been used in assessing population exposure to environment tobacco smoke (ETS) for the purpose of assessing risk estimates for lung cancer related to ETS exposure (Thompson et al.,
1990; Repace and Lowrey, 1993). Total plasma cotinine concentration is therefore determined among chewers and non-chewers.

Owing to these, the study conducted with the aims to find out the cytogenetic biomarkers and exposure marker along with level of zinc and copper in the serum of chewers and non-chewers. Also, the role of chewing areca nut and tobacco as well as the mixture of these compounds in the genesis of oral sub mucous fibrosis has also been studied considering the cytogenetic endpoints.