Introduction
It is known that a number of factors are associated with causation of cancer such as environmental, occupational, dietary, lifestyle along with other host factors such as immune, genetic or viral factors. Certain cancer such as Burkitt Lymphoma and nasopharyngeal carcinoma are reported to be associated with Epstein Barr virus along with other environmental, dietary and genetic factors. It is reported that the human population is exposed to the number of chemical mutagens accidentally, occupationally, dietary or by lifestyle factors. Almost 80-90% of cancers are attributable to such factors (Trivedi et al., 1996). Cancer is a multi-factorial, multi-faceted and multi-mechanistic disease requiring a multi-dimensional approach for its diagnosis, treatment and prevention. Worldwide, there were estimated 10.1 million new cases, 6.2 million deaths and 22.4 million persons living with cancer in the year 2000 and the number of new cases is expected to grow by 50% over the next 20 years, to reach 15 million by 2020 (Bernard and Paul, 2003).

Oral cancer cases in India frequently presented with local or regional metastasis at the time of diagnosis. The overall survival rate for patients with oral cancer is lowest (less than 50%) and has not changed during the past two decades (Nagler, 2002). Oral cancer is the sixth most frequent cancer in the world accounting 3,00,000 new cases annually. The incidence of oral cancer is comparatively very low in Western countries, which is 2-6% of all malignancies (NCRP, 1996). While in India, it constitutes nearly a third of all cancer cases. Annually, 75,000 – 80,000 new oral cancer cases are registered in India (NCRP, 1996). Various cancer registries have documented that the high incidence of oral cancer is due to widespread habits of tobacco
chewing and smoking in Indian population (Gupta and Nandkumar, 1999). These differences in the incidence of oral cancer between Western countries and India may be due to difference in the habit of tobacco usage. The Gujarat Cancer and Research Institute, Ahmedabad (India) registers about 1700 new cases of oral cancer every year (GCRI, 2001). Presently, incidence of oral cancer in India for both sexes is highest reported worldwide (Parkin et al., 1997). Oral sub mucous fibrosis (OSMF) is an insidious, chronic fibrotic change affecting any part of oral mucosa and has been considered as an oral precancerous condition (Pindborg, 1968; WHO, 1984). OSMF is a chronic disease of oral mucosa characterized by inflammation and progressive fibrosis of the lamina propria and deeper connective tissues, followed by stiffening of mucosa resulting in difficulty in mouth opening. Symptoms of OSMF include localized burning sensation and intolerance to spicy food, followed by ulceration and blanching of the mucosa and the formation of characteristic fibrous band (Pillai et al., 1992). Higher prevalence of oral sub-mucous fibrosis (OSMF), oral leucoplakia and oral cancer is suspected to be associated with the habits of tobacco with/ without areca nut chewing. The association of tobacco-betel quids with oral cancer has been shown to be particularly strong from various studies in India as early as 1895 (Babu, 2001). The significant observations were that the oral cancer had a strong association with betel-tobacco quids chewing and tobacco smoking/ chewing had an additive effect. Several subsequent studies reached similar conclusions (Krishnamurthi and Shantha, 1994).

Areca nut, seed of the fruit of the oriental palm, *Areca catechu* is the basic ingredient of a variety of widely used chewed products, which is more commonly named as betel nut. Areca nut is the fourth most commonly abused substance after nicotine, ethanol and caffeine (Warnakulasuriya and Peters, 2002). It also has addictive potential and reported to possess cytotoxic, mutagenic and genotoxic properties (Wary and Sharan, 1988; IARC, 2004). Lime (one of the ingredient of quid with tobacco and areca nut) has also been considered to play an important role in the genesis of oral cancer (Tanaka et al., 1983). Tobacco is known to be the most addictive substance and
consumed mainly by smoking and chewing. The tobacco smoke contains several groups of carcinogens including polynuclear aromatic hydrocarbons (PAHs), heterocyclic hydrocarbons, volatile hydrocarbons, nitrohydrocarbons, aromatic amines, N-heterocyclic amines, N-nitrosamines, aldehydes, miscellaneous organic compounds, inorganic compounds and phenolic compounds (IARC, 2004). Globally nearly 5.4 million persons die every year as a consequence of tobacco smoking, with three quarters of all deaths currently occurring among men (Mathers et al., 2006). Tobacco smoking (cigarette) is more prevalent in Western countries; while tobacco chewing, smoking ( bidi) and snuffing along with other ingredients like, betel nut, lime, catechu etc. are the most prevalent habits in India (Jeng et al., 2001). Due to these major etiological factors and increasing incidence rate it is suggested that oral cancer in India should be considered as a “new epidemic” (Gupta, 1999).

Betel quids with tobacco are used in Central East, South and Southeast Asia, in the Western Pacific and in migrant communities (Bhonsle et al., 1992; Gupta and Ray, 2002). Twenty-eight carcinogens have been identified in smokeless tobacco. The major and most abundant groups of carcinogens are the non-volatile alkaloid-derived Tobacco-specific N-nitrosamines (TSNAs) and N-nitrosoamino acids. Other carcinogens reportedly present in smokeless tobacco include volatile N-nitrosamines, certain volatile aldehydes, traces of some polynuclear aromatic hydrocarbons such as benzo[a]pyrene, certain lactones, urethane, metals, polonium-210 and uranium-235 and -238 (Brunnemann and Hoffmann, 1992). There are three major types of nitroso compounds in smokeless tobacco; a) nonvolatile TSNA, including 4-(metylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosornornicotine (NNN); b) N-nitrosamino acids, including N-nitrososarcosine (NSAR), 3-(metylnitrosamino)propionic acids (MNPA) and 4-(metylnitrosamino)butyric acids (MNBA); and c) volatile N-nitrosamines, including N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPRY), N-nitrosopiperidine (NPIP) and N-nitrosomorpholine (NMOR) (IARC, 2007). The oral use of smokeless tobacco is widely prevalent in India and various
methods of consumption include chewing, applying tobacco preparations to the teeth and gums. In fact, there are several types of chewing habit featuring the use of betel quid (fresh betel leaf, fresh areca nut, slaked lime, catechu with or without tobacco), tobacco with lime (*Khaini*), *Mawa* (mixture of areca nut, tobacco and slaked lime), Pan masala plain (areca nut, catechu, lime, cardamom and unspecified flavoring agents etc.) and pan masala with tobacco (*gutkha*).

Nicotine in tobacco is rapidly absorbed from the oral cavity and from the gastro-intestinal tract after swallowing, but less rapidly than from cigarette smoke (Stratton *et al.*, 2001). The pH of areca nut and tobacco chewing in solution is a significant factor for nicotine bioavailability and thereby increase in pH lead to increases in nicotine blood concentrations (Fant *et al.*, 1999). Nicotine has been reported to be associated with a number of cellular effects in various *in vitro* systems, often demonstrated at much higher concentrations than those achieved after smokeless tobacco product use. Many of these effects are related to binding and activation of nicotinic acetylcholine receptors in non-nervous tissues. Nicotine may lead to redistribution of receptor subunits in cell membranes resulting in downstream alterations of signalling involved in cellular proliferation and apoptosis (Buisson and Bertrand, 2002). Nicotine is extensively metabolized, with cotinine as the main primary metabolite and its metabolic products are chiefly excreted via kidney. (Hukkanen *et al.*, 2005). A main pathway of nicotine metabolism is shown in Fig. 1. Cotinine, the major proximate metabolite of nicotine, has been widely used as a biomarker of tobacco exposure (Benowitz, 1983; Etzel, 1990). Cotinine levels may represent an alternative measure of tobacco exposure to complement and analysis of such biomarkers of tobacco research has increased due to health risks associated with tobacco exposure. Cotinine is the preferred serum/plasma biomarker for tobacco exposure.
It has been known that free radicals produced during auto-oxidation of polyphenols in saliva of the tobacco users are crucial in the initiation and promotion of oral cancer (Jeng et al., 2001). N-nitroso compounds constitute the most abundant carcinogens present in tobacco, with tobacco-specific nitrosamines (TSNA), representing an important class of genotoxic carcinogens (Hecht and Hoffman, 1988). The TSNA are metabolically activated to yield electrophiles, which react with cellular components, including nucleophilic centers of DNA leading to DNA damages (Loechler et al., 1984). Thus, there is continuous endogenous damage to cellular DNA by free radicals and accumulation of such damage has been found to play a significant role in carcinogenesis (Toyokuni et al., 1995). A large number of genotoxicity tests are available for use in hazard evaluation that detects the two main categories of mutations, gene mutation and chromosomal aberration, as well as indications of DNA damage. Tests to assess these endpoints can be carried out both in vitro and in vivo. In order to assess adequately any expression of genotoxicity, a simplified systematic approach to the selection of these tests is required. There must be an ordered approach using a limited number of well-defined tests that complement each other in terms of endpoints and that permits a systematic assessment of genotoxicity (Mutagenicity Guidelines, 1993).

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 as well as occupational exposure to potentially mutagenic and/or carcinogenic chemicals. The buccal micronucleus cytome (BMCyt) assay is increasingly used in molecular epidemiological studies for investigating the impact of nutrition, lifestyle factors, genotoxic exposure on DNA damage and genotype, chromosome instability and cell death. The biomarkers measured in this assay have been associated with increased risk of accelerated ageing, cancer and neurodegenerative diseases (Thomas et al., 2009). The BMCyt assay is a minimally invasive means of investigating events that identify changes in potential biomarkers that are reflective of DNA damage (MN and/or nuclear buds), cellular proliferation potential (binucleated cells) and/or cell death parameters (condensed chromatin, karyorrhectic, pyknotic and karyolytic cells). These changes show distinct differences between the cytome profiles within normal ageing relative to that for premature ageing clinical outcomes such as cancer. In light of the fact that over 90% of cancers are epithelial in origin and that buccal mucosa is the site for oral cancer, buccal cell utilization has great epidemiologic potential as a means for genotoxic and cancer risk assessment (Cairns, 1975).

Chromosomal aberrations are departures from the normal set of chromosomes either for an individual or a species. They can refer to changes in the number of chromosomes sets, changes in the number of individual chromosomes or changes in appearance of individual chromosomes through mutation-induced rearrangements. They can be associated with genetic diseases or with species differences. The chromosomal aberration assay in cultured cells has been widely used for many years and it has been proved to be a useful and sensitive test for detection of genotoxic agents. Tests are carried out with and without extrinsic metabolic activation (Galloway, 2000).

The blood cytome assay has evolved into a comprehensive method for measuring chromosome breakage, DNA misrepair, chromosome loss, nondisjunction, necrosis, apoptosis and cytostasis. This method is now also used to measure nucleoplasmic bridge, a biomarker of dicentric chromosomes.
resulting from telomere end-fusions or DNA misrepair and to measure N buds, a biomarker of gene amplification. The “cytome” concept implies that every cell in the system studied is scored cytologically for its viability status (necrosis, apoptosis), its mitotic status (mononucleated, binucleated, multinucleated) and its chromosomal damage or instability status. For these reasons, it is now appropriate to refer to this technique as the Cytokinesis block micronucleus cytome assay (Fenech, 2007). Dave et al., (1991) demonstrated statistically significant increase in chromosomal aberrations and micronucleated cells in the peripheral blood of pan masala chewers. Earlier Schmid (1975) and Heddle (1973) also suggested that a simpler approach to assess chromosome damage in vivo was to measure micronuclei (MN).

O’stling and Johanson (1984) were the first to develop a microgel electrophoresis technique for detecting DNA damage at the level of single cell. In their technique, cells embedded in agarose were placed on a microscope slide, the cells were lysed by detergents and high salt and the liberated DNA electrophoresed under neutral conditions. The neutral conditions used greatly limited the general utility of the assay. Subsequently, Singh et al., (1988) introduced a microgel technique involving electrophoresis under alkaline (pH 13) conditions for detecting DNA damage in single cell. At this pH, increased DNA migration is associated with increased levels of frank single-stranded break (SSB), SSB associated with incomplete excision repair sites and alkali-labile sites (ALS). Two years later, Olive et al., (1990) introduced another alkaline version of this assay in which DNA is electrophoresed at a pH 12.3. Since the introduction of the alkaline (pH-13) comet assay in 1988, the breadth of applications and the number of investigators using this technique have increased almost exponentially.

FISH (fluorescence in situ hybridization) is a cytogenetic technique that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity. FISH is often used for finding specific features in DNA for
use in genetic counselling, medicine and species identification as well as to detect and localize specific mRNAs within tissue samples. In this context, it can help to define the spatial-temporal patterns of gene expression within cells and tissues (Jeffrey et al., 2003). It has been observed that the gene most frequently mutated in human cancer is p53 (Harris, 1996; Sakai and Tsuchida, 1992). The tumor suppressor gene p53, known as the guardian of the cell cycle, is the most common target for mutations observed in several malignancies such as oral cancer, gastric carcinoma and cervical cancer. A highly relevant chromosomal region in carcinogenesis is the short arm of chromosome 17, where the p53 gene is located (band 17p13.1). The wild-type p53 gene is a tumour suppressor gene coding for a nuclear phosphoprotein that plays a crucial role in the cellular response to DNA damage by inducing cells to arrest in G1 or enter into apoptotic cell death resulting in the failure to express functional p53 protein (Kastan et al., 1991; 1992). In this context, it is important to develop new biomarkers of genetic damage with special relevance in terms of cancer induction. The application of emerging molecular cytogenetic methods in biologically based risk assessment may help to clarify the uncertainties of risk of chewing habit.

The most commonly observed fibrotic changes were seen in buccal mucosa and palatal mucosa in OSMF. However, only a fraction of the betel nut chewers develop OSMF suggesting genetic susceptibility or lack of anti-fibrotic mechanism in developing OSMF. In addition, potential role of copper in causation of OSMF could also be considered as areca nut contains appreciable amount of copper and soluble copper, released in the mouth while chewing areca nut. Copper is implicated in tissues fibrogenesis via copper dependent enzyme (lysyl oxidase), which is crucial in stimulating fibroblasts in oral submucous fibrosis leading to excessive cross-linking and accumulation of collagen (Ma et al., 1995). The regeneration of the oral mucosa is dependent on zinc and vitamin A. Earlier, Jayadeep et al., (1997) also reported a significantly decreased zinc level only in male patients with leukoplakia and sub mucous cell carcinoma.
Thus in this study, the cytogenetic alterations in the target tissue as well as peripheral blood lymphocytes among the chewers, chewers with oral sub mucous fibrosis and non-chewers were evaluated in order to assess the genotoxic potential of areca nut and tobacco. Further, the cytogenetic endpoints have been used as biomarkers of effect which may allow a reasonable epidemiological evaluation in predicting the cancer genesis.