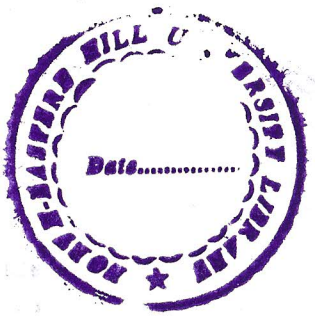
Extent of DNA damage induction by raw betel-nut extract in mammalian cells and loss of heterozygosity at chromosome 9p in oral and esophageal carcinoma in raw betel-nut chewers.



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DECLARATION

I, Allen J. Freddy, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the North Eastern Hill University for the degree of Doctor of Philosophy in Zoology.

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ABBREVIATIONS

AAS	Atomic absorption spectrometer
AEBN	Aqueous extract of betel nut
AGT	Average generation time
APS	Ammonium persulphate
ARC	Arecoline
ARF	Alternate reading frame
ASNAs	Areca nut specific nitrosamines
BMC	Bone marrow cells
BN	Betel nut
BNE	Betel nut extract
BQ	Betel quid
BSO	L-Buthionine-S-R-Sulphoximine
BUdR	5-Bromodeoxyuridine
CAs	Chromosomal aberrations
CDKN2A	Cyclin dependant kinase 2A
CDK's	Cyclin-dependent kinases
CIN	Chromosomal instability
CKI	Cyclin dependant kinase inhibitor
DNA	Deoxyribonucleic acid
dNTP	di-nucleotide triphosphate
DTT	1, 4- Ditheithretol
EC	Esophageal carcinoma
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
FPG	Fluoresce plus Giemsa staining
GSH	Glutathione
HNSCC	Head and neck squamous cell carcinoma
HPBLs	Human peripheral blood lymphocytes
i.p	Intra peritoneal
INK4	Inhibitor of kinase 4
KCl	Potassium chloride
LOH	Loss of heterozygosity
MI	Mitotic index
MNPN	3-(methyl-N-nitrosamino) propionitrile
MSI	Microsatellite instability
MTS1	Multiple tumor suppressor-1
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NET	Sodium chloride, ethylene diamine tetra acetic acid, tris

NG	N-nitrosoguvacoline
OSCC	Oral squamous cell carcinoma
OSF	Oral submucous fibrosis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNK	Polynucleotide kinase
Rb	Retinoblastoma
ROS	Reactive oxygen species
RPMI	Rosewell park memorial institute
SCC	Squamous cell carcinoma
SCE	Sister chromatid exchange
SOD	Super oxide dismutase
STRs	Short tandem repeats
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris, ethylene diamine tetra acetic acid
TSG	Tumor suppressor gene

GENERAL INTRODUCTION

Environment encompasses all non-genetic factors such as diet, lifestyle and infectious agents. In this broad sense, the environment is implicated in the causation of the majority of human cancers, as has been demonstrated since the 1960s (Doll, 1969). Such a broad meaning of the word environment is assumed when referring to gene environment interactions. On the other hand, environmental factors can include only the (natural or man-made) agents and circumstances encountered by humans in their daily life, upon which they have no or limited personal control. In this context, environmental factors are restricted to air, water, soil and food pollutants, including physical pollutants such as sources of ionizing radiation. These ambiguities in the terminology and the inconsistencies in the use of the vocabulary by cancer researchers contribute to public confusion regarding the role of environment in cancer. A distinction relevant to cancer prevention may be made between factors related to personal behaviors, lifestyle (e.g. tobacco smoking, chewing of betel quid and alcohol drinking), involuntary exposures, such as those linked to air, water, soil or food pollutants, and occupation. It would be preferable to abandon the term environment and to use terms such as non-genetic or modifiable determinants of disease (broad sense of environment) and pollutants (narrow sense). The oral cavity and esophagus combined is the sixth commonest site of cancer in both sexes. In many countries the mortality rate is increasing among younger men. A causal role in the etiology of both oral and esophageal cancer has been established for tobacco, both smoking and chewing, separately and in conjunction with betel quid chewing; with alcohol consumption and, less certainly, with other factors such as poor oral hygiene, nutritional factors and certain occupational exposures. In Western countries, there is convincing evidence that a large attributable risk can be ascribed to the joint habits of cigarette smoking and alcohol consumption. In Asian countries, a high attributable risk can be ascribed to cigarette smoking and betel-nut chewing. Cancer of the oral cavity is an important form of cancer and one for which practical prospects for prevention already exists. However the prognosis of esophageal cancer is very poor, its 5 years survival rate is only about 10% for the patients at late or advanced stage. The early characteristic of the

subjects predisposed to esophageal cancer is the abnormal proliferation of epithelial cells, morphologically manifested as basal cell hyperplasia, dysplasia and carcinoma in situ, which could be considered as precancerous lesions of esophageal cancer.

Related to its many functions, the oral cavity contains several different types of stratified squamous epithelia, including those classified as nonkeratinized, parakeratinized, and orthokeratinized (Burkhart and Maerker 1981). Regional variation and heterogeneity within each type of epithelium also include glandular epithelium (salivary glands) and taste buds, the latter on the dorsal and lateral tongue. Primarily nonkeratinized epithelium provides a lining in the cheeks, lips, floor of mouth, ventral aspect of the tongue, soft palate, and upper and lower vestibular sulci. Parakeratinized and orthokeratinized epithelium lines the hard palate and the mucosa that surrounds the teeth (attached gingiva). The dorsal tongue and gingival margin are such zones. The basement membrane zone, the papilla and reticular zones of the lamina propria, and beneath these, the submucosa, typically support the various oral epithelia. The very similar structure of the oral epithelium and the epidermis, including the squamous nature of both and the generation of a surface barrier, naturally implies that many of the research results with epidermal keratinocytes are also applicable to the oral epithelium.

Damage to genetic material could result in a number of immediate and long-term effects, finally leading to cancer. A search for possible voluntary exposure that could be used as a model for investigating human carcinogenesis led to consideration of betel nut chewing. Investigations of this possibility offer an opportunity both to study this specific hypothesis, investigate and to develop approaches for similar studies of other exposures along with betel nut. This approach could be further described as the use of laboratory tests to define exposures, susceptibility factors, and the pathophysiological sequence of events linking exposure and disease in epidemiologic studies of chronic diseases. Carcinogenesis studies in experimental animal models indicate that an early event for many of the chemical carcinogens is to cause damage to the genetic material. Oral cancer is one of the most prevalent cancers in South and Southeast Asian

countries. There are strong indications for an association of the habit of betel quid chewing with cancers of the mouth, oropharyngeal cavity, and upper parts of the digestive tract (Dave et al., 1992; IARC Monograph 1985). Moreover, chewing and smoking habits interact synergistically for these cancers (Jayant et al., 1997). Although some pathological (Pindborg, 1980), epidemiological (Hirayama 1966) and genetic studies on oral cancer and precancer have been previously reported, the incidence of oral cancer is still very high in these countries and only a few efforts have been carried out for its prevention.

Oral cancer was described in the Sushruta Samhita, a treatise on Indian surgery written in Sanskrit around 600 B.C. Literary references to the habit of chewing betel quid (betel leaf, areca nut and lime) in India are at least 2,000 years old. Tobacco was introduced around the sixteenth century. It is estimated that at least 600 million individuals consume areca nuts in one form or another worldwide. The habit is now widespread in Southeast Asia and the South Pacific islands and in people of Indian origin elsewhere in the world. The betel quid chewing habit is found all over the world wherever Indians have settled. The major areca nut alkaloids are arecoline, arecaidine, arecolidine, guvacoline and guvacine (IARC Monograph, 1985). Arecoline (1,2,4,5,-tetrahydro-1-methyl-pyridine- carboxylic acid; molecular weight 155.19) is the most abundant alkaloid of areca. These alkaloids undergo nitrosation and give rise to N-nitrosamines (Hoffmann et al., 1994). It has been suggested that metabolic activation may involve the cytochrome p450 system (Sundqvist et al., 1991; Wary and Sharan, 1991). The nitrosation of arecoline may produce a variety of betel quid-specific nitrosamines which may interact with DNA, proteins or other targets forming adduct to exert its carcinogenic activity. The introduction of tobacco from European countries reinforced this practice, and now almost all habitually chewed betel quid include tobacco. A comparison of the carcinogenicity of the habit of chewing betel quid with and without lime has been attempted through a reassessment of the available epidemiological evidence on the etiology of oral cancer and pre-cancer. Slaked lime included in betel quid causes inflammation in the sub mucosal area. Calcium hydroxide content of lime in the presence of the areca nut is primarily responsible

for the formation of reactive oxygen species that might cause oxidative damage in the DNA of buccal mucosa cells in betel quid chewers (Nair et al., 1990).

Ever since the discovery of glutathione (GSH) in the early thirties, this tripeptide has been a subject of continual interest. This versatile antioxidant is found to be involved in various biological functions (Meister, 1983). Although this tripeptide can exist in both reduced (sulphydryl) and oxidized (disulphide) form, it is maintained *in vivo* predominantly in the former state, however the, balance is maintained through the action of equally ubiquitous enzyme Glutathione Reductase (Knox, 1960). GSH is found throughout the cell, with the bulk in cytoplasm, subcellular particles, such as nucleus, mitochondria having smaller amounts (Bigalow and Tutle 1993). It plays an important role in regulation of cellular proliferation and cellular defense against radiation (Chatterjee and Jacob-Raman 1986) and various xenobiotics (Syng-ai et al., 2002). Most workers have used BSO for studies involving the modulation of GSH levels in cells. BSO inhibits GSH synthesis and very low levels of GSH can be obtained over a relatively short period without appreciable toxicity. The most useful approach to deplete the level of endogenous GSH is the treatment with BSO, a specific inhibitor of γ-glutamylcysteine syhthetase, the enzyme that catalyzes the step of GSH synthesis (Griffith and Meister 1978). BSO is a potent selective inhibitor of GSH synthesis that is highly effective both *in vivo* and *in vitro* without showing any side effect (Griffith and Meister 1978). Depletion of GSH by treatment with BSO sensitizes the cells to toxic effects of heavy metals (Singhal et al., 1987, Naganuma et al., 1990), nitrogen mustard (Suzakake et al., 1982, Suzakake et al., 1983), radiation and cisplatin (Edgren and Revez 1987) but not radiomimetic drugs like bleomycin (Chatterjee et al., 1989).

The precancerous lesions are instable, they can develop to cancer, or stay for a couple of years without any changes, or even return to normal. What is the most important factor to decide the precancerous lesions to developing different directions, especially in those with a similar morphology? What is the key point to induce mild precancerous lesions to develop cancer? Our assumption is that there

exist different molecular changes in precancerous lesions with a similar morphology. To characterize the molecular changes in carcinogenesis of both oral and esophageal cancers, the mechanism of these cancers could be elucidated and the biomarkers for early diagnosis and mass survey of high-risk populations could be established. It has been shown that heavy chewers of betel-nut (BN) in the Meghalaya state show higher DNA damage, delay in cell kinetics, p53 expression and lower GSH-level than non-chewers (Kumpawat and Chatterjee 2003).

The north-east Indian variety of BN is raw, wet and consumed unprocessed and chewed along with betel-leaf and slaked lime. The constituents of this nut show higher alkaloids, polyphenol and tannins compared to the dried one (Sharan 1996) and is locally known as 'Kwai'. (Stich et al., 1983) demonstrated the genotoxic potentiality of saliva of kwai chewers of the tribal population of Meghalaya state of the north-eastern region of India in Chinese hamster ovary cells. The average age of onset of chewing among tribes was about 12 years, and at 35 years of age and older, the frequency of oral carcinoma rose significantly.

Microsatellites are repeated DNA sequences scattered widely within the genomes and closely linked with many important genes (Shamoo, 2003). In recent years many researchers have indicated that the alteration of microsatellite DNA is one of the important markers, which could induce normal cells to undergo immortal and neoplastic transformations. It was reported that an extensive loss of microsatellite DNA was discovered in many tumors such as colon cancer. Some microsatellite loci often exist in the hot spots of loss of heterozygosity at a high frequency in some specific malignancies. Tumor suppressor genes, which are associated with the development and progression of tumors, may harbor in the vicinity of these hot spots.

Histology of the tumor samples, the current gold standard for assessing the risk, is reasonably effective in judging the malignant risk of high-grade pre-invasive lesions. It is, however, a poor predictor for lesions without dysplasia, or with minimal dysplasia, as only a few of these lesions will progress to cancer. This poses an enormous dilemma for clinicians as to whether these lesions should be aggressively treated or not. Recent studies show that loss of specific chromosomal

regions that contain known or presumptive tumor suppressor genes is an early predictor of subsequent progression of oral premalignant lesions. Incorporation of loss of heterozygosity (LOH) findings into staging of oral premalignancy could improve our ability to identify and manage high-risk premalignant lesions, particularly those with relatively benign histology but high-risk genetic changes (high-risk LOH pattern).

It is true that from the background we have some idea about the mechanism of action of the betel-nut extract; however, we will have to know some more things which will be helpful to understand about its action. Therefore, we have primarily two objectives to investigate and they are:

- Evaluation of the extent of initial DNA damage / lesions induced by raw betel nut in mammalian cells.

- Identification of critical regions of Loss of Heterozygosity (LOH) by deletion mapping by PCR based techniques using appropriate microsatellite markers in 9p chromosome arm in order to know the status of p16 gene in tumor samples collected from raw-BNE chewers.

Some pictures of ① betel nut, leaf BQ. etc. & ② cancerous lesions (histology) would have been suitably included!