SUMMARY

Areca nut or betel-nut is the seed of a tall, slender areca palm (*Areca catechu*), native to the fringes of the Indian and west Pacific Oceans. It is thought to have originated on the Malay Peninsula. Areca nut is one of the most widely used psychoactive substances with several hundred million users worldwide, predominantly in southern Asia. It has been estimated that approximately 600 million people in South-east Asian countries are addicted to areca. Betel users discovered that the addition of lime helps to extract the vital essence of the nut; the catalytic lime is either powder (calcium oxide) or paste (calcium hydroxide). The International Agency for Research on Cancer (IARC) regards betel nut to be a known human carcinogen. In countries and communities where betel is consumed extensively, oral cancer forms up to 50% of malignant cancers. Betel nut chewing is linked to the development of oral [Zhang and Reichart, 2007] and esophageal cancer [Wu et al., 2006]. Betel-nut chewers with faulty gene have higher risk of mouth cancer. Oral squamous cell cancer is the most common malignant tumor caused by chewing of betel nut. Powdered slaked lime applied to the chewed Areca nut with Piper betle inflorescence while chewing causes pH to rise to approximately 10, at which reactive oxygen species are generated from betel quid ingredients in vitro. Reactive oxygen species, together with sustained lime-induced cell proliferation, suggest a possible mechanism of carcinogenesis for this tumor. This causes the betel quid ingredients to generate free radicals which, together with carcinogens from the tobacco smoke, cause DNA damage that cannot be repaired because of the rapid cell turnover caused by the caustic lime. The evidence for the carcinogenicity of humans to betel quid with tobacco was evaluated as sufficient; the
evidence for betel quid without tobacco was evaluated as inadequate. Epidemiological studies have elucidated that the habit of chewing betel quid (BQ) is associated with an increased risk of oral submucous fibrosis (OSF) and oral cancer. The Khasi’s in Meghalaya chew one quarter of the betel nut along with a portion of the betel leaf on which lime has been smeared. As a rule, the inclusion of tobacco and spices or other perfumes are avoided. This unique situation favors an investigation into the length and exposure of the oral mucosa to the chemicals released from the BNE and leaf during the course of the day (Stich et al., 1983). East Khasi Hills a district in Meghalaya of the north-eastern region of India has one of the highest incidence rates of oral, oropharyngeal and esophageal cancer which are of particular concern. Studies reveal that Head and neck cancers still have a high mortality rate. Epidemiological evidence from case-control studies of Head and neck squamous cell carcinoma (HNSCC) indicates that a family history of Head and Neck cancer is also a risk factor. Lip cancer is among the sites that show the strongest cancer clustering within families [Cannon-Albright et al., 1994]. Cancers occur as a consequence of alterations to genes that control growth and differentiation. Tumor suppressor genes (TSGs) are present in all cells, and serve to regulate normal proliferation, or mediate cell-specific differentiation. Using simple PCR based techniques, one can identify if there is loss of genetic material, represented by complete deletion, or loss, of one allele (also known as loss of heterozygosity or LOH). LOH is frequently observed in a variety of human cancers, and regions with frequent LOH may contain tumor suppressor genes. In addition, LOH may associate with the regions affected by haplo-insufficiency of a group of genes. Thus, detection of LOH will likely remain a cornerstone for predicting tumor aggressiveness for many human tumors [Maris et al., 2005]. Today,
epigenetics refers to the study of heritable changes in gene expression without the change in gene sequence. Abnormal methylation of DNA may result in increased transcription of oncogenes or silencing of tumor suppressor genes and is common in a variety of human cancer cells [Esteller, 2005]. Although the ramifications of global hypomethylation for tumor development are less well understood, it might contribute to chromosomal instability and then increases in gene expression [Baylin et al., 2006]. Betel nut chewing with or without tobacco has been shown to be independently associated with the development of esophageal cancer in Assam [Phukan et al., 2001]. Furthermore, LOH could also be observed in neoplastic and apparent phenotypically normal preneoplastic cells that may eventually progress to become cancer [Pan et al., 2005]. The hypermethylation of CpG islands in gene promoter regions is associated with aberrant silencing of transcription and has been regarded as a common mechanism for inactivation of tumor suppressor genes in human cancer [Herman et al., 2003]. As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns [Baylin et al., 2000]. Loss of the INK4a/ARF locus results in deregulation of both, p53- and pRB- pathways and hence uncontrolled cell proliferation [Lal et al., 2008]. Approximately 50% OSCC tumors stain positive for TP53. The Rb gene, located on chromosome 13q14.2, was the first tumor suppressor gene to be identified in humans and was initially determined to be associated with the development of retinoblastoma [Friend et al., 1986]. Suppression of pRb function through hyperphosphorylation causes the release of the E2F factors and triggers a burst of gene expressions that facilitates G1-S transition [Buchkovich et al., 1989; Johnson, et al., 1993]. Functional loss of the Rb gene frequently occurs in the carcinogenic processes of many types of cancer [Benedict et al., 1990]. LOH at the Rb
locus is an important event reflecting potential functional alteration in the \( Rb \) gene. LOH on 13q, where \( Rb \) is located, is a common feature in many types of cancer involving bladder, lung, breast, head and neck, and other organs. In human esophageal cancer, LOH at the \( Rb \) locus was observed in 54% of SCCs and in 36% of adenocarcinomas [Huang et al., 1992]. Studies of genetic progression have suggested that p53 alteration occurs at greater frequency in invasive carcinomas than in noninvasive lesions [Boyle et al., 1993]. Several studies have demonstrated that 40–70% of SCCHN contain mutations in exons 5–9 at the p53 locus [Shin et al., 1996]. One of the earliest known events in head and neck squamous cell carcinogenesis may occur at chromosome 9p21. This chromosome contains the locus for two proteins, ARF and p16. Inactivation of p16 may occur through several different mechanisms including homozygous deletion, methylation of the gene promoter with subsequent transcriptional silencing, and single base pair mutation. The most frequent method of inactivation is homozygous deletion.

An association between aberrant pRb and p53 expression was observed in bladder and several other types of cancer [Cordon-Cardo et al., 1997; Cote et al., 1998]. Loss of \( Rb \) can activate apoptosis at least in part via elevated p53 expression, and loss of \( p53 \) gene function prevents cell death in the central nervous system of \( Rb \)-mutated mouse embryos [Macleod et al., 1996]. It was reported that betel quid chewing with or without tobacco is associated with the development of esophageal cancer in Assam [Phukan et al., 2001]. In the Indian population, a great majority of OSCCs and ESCCs are associated with chronic tobacco consumption. Due to this fact, it has been difficult to assess the effects of purely and predominantly RBN induced genetic alterations without interference of other confounding factors like tobacco smoking or chewing.

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Therefore, present study aimed for determining whether RBN alone can induce these cancers and if yes, then are the above mentioned genes involved in the development of these cancers?

Thus the present study was designed to understand the role of raw betel-nut (RBN) in ESCC and OSCC development. We analyzed ninety nine oral and esophageal cancer samples for LOH by using PCR based technique of deletion mapping for 10, 5, and 3 highly polymorphic microsatellite markers on 9p21-23, 13q12-14, and 17p12-13 chromosome arms, respectively. The samples were also assayed by Methylation specific PCR to detect the promoter hypermethylation of p16INK4a and RB1 genes.

The following conclusions are drawn from the present study design:

- The study revealed that Esophagus is the predominant site of cancer development in RBN associated carcinogenesis. The oral cavity is the site which is exposed mainly to the chewed components of RBN. It has been observed that the RBN chewers mostly swallow the chewed RBN, may be due to this practice the esophagus is getting exposed to the carcinogenic constituents of RBN. It is reported earlier that in the presence of saliva the genotoxic ability of areca nut alkaloids increases. This may be the probable reason for development of esophageal cancer more compared to oral cavity ones.

- The LOH frequency of 9p21-23 region was observed in 69% [68/99] of cases. In ESCC cases [N=62], 72% (N=45) samples had LOH at 9p21-23, whereas 57% [22/37] samples of OSCC had demonstrated LOH for this arm. Of the Ninety nine samples, 32 samples had LOH only on 9p. Sixteen samples
showed LOH for 9p + 13q whereas 19 samples showed LOH for 9p+ 13q + 17p. For 9p + 17p, LOH was observed for 10 samples. But 22 samples did not LOH for any of the marker of the three chromosomes 9p, 13q and 17p. This indicates that the LOH of 9p21-23 region plays an important role in the initiation and progression of oral and esophageal cancer among RBN chewers with and without tobacco habit. The minimal region of deletion detected in oral and esophageal cancer are 9p23 [2cM,] 9p21 (p16INK4a locus) [1cM], and 9p21 distal [4cM].

- In oral cancer cases those with only RBN chewing habit has higher frequency of LOH of 9p23 region [57%] along with 9p21 [57%] but in RBN + tobacco habit patients only 9p21 regions had shown higher LOH frequency but not the 9p23 region. Some of the hyperplasia /dysplasia samples had also shown LOH of 9p21 and 9p23 regions. These observations suggest that 9p23 region is harbouring some tumor suppressor genes which may have role in the initiation as well as progression of RBN chewing associated oral cancer. One of the tumor suppressor genes present in 9p23 region our study is protein tyrosine phosphatase, receptor type, D gene [PTPRD]. The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation.

- There had been statistically significant association observed between age and LOH frequencies in esophageal cancer cases of only RBN chewing habit. In RBN +tobacco habit cases also had shown similar statistically significant
association. This suggests that the >50yrs of age compared to <50yrs with same histopathology, has higher LOH frequencies on the same as well as multiple chromosome arms. Such microsatellite markers can be of value in screening patients of >50yrs age prognosis and overall survival. As there was no follow up mechanism for cancer patients, so could not have data about their survival.

- The 13q14 chromosome arm had lower LOH frequency for oral cancer samples. The LOH frequency was predominantly found in esophageal cancer cases. This region harbours RB1, gene which is an important tumour suppressor gene and its inactivation had been reported in wide array of human cancers. The data suggests that RB1 gene may have a limited role in RBN associated OSCC but it may have a bigger role in the progression of RBN induced ESCC because the higher LOH frequency of 13q14 was found in the advanced grade (SCC) samples of esophageal cancer.

- The 17p13 chromosome arm, which harbours p53 gene, had shown lower LOH frequency compared to 9p and 13q arms. This indicates that p53 gene locus may have limited activity in RBN associated OSCC and ESCC cases. It might be involved during the advanced stage of these cancers.

- A total of 22 samples did not show any LOH for the three chromosome arms in this study which suggest that these samples may have LOH on other frequently deleted chromosome arms or on the other regions of the present study chromosome arms. Further, we need to the study the involvement of other chromosome arms such 3p, 8p, 11q etc in RBN induced cancers to better understand the role of genetic alterations like LOH in these cancers.
The promoter hypermethylation of p16INK4a gene was detected in 30% [14/42] sample and RB1 gene had only 11% methylation [3/26]. In only RBN chewing patient samples p16INK4a methylation was predominant compared to RBN+Tobacco habit cases. The hyperplasia /dysplasia samples had also shown the methylation of only p16INK4a gene. This suggests that p16INK4a methylation may be involved in the initiation and progression events of ESCC and OSCC. But 32 samples did not show methylation for the two genes of this study. In future we need to analyze the promoter hypermethylation of other genes such as RASSF1A, MGMT, DAPK, in which methylation is an important event leading to their inactivation.

Together the alteration of p16INK4a gene locus by LOH and promoter hypermethylation is found to be approx 75%. This observation indicates that p16INK4a-RB pathway alterations may be playing an important role in RBN associated OSCC and ESCC development.

This study has provided us with better understanding about LOH of 9p, 13q, 17p and p16INK4a, RB1 gene promoter methylation events in only RBN chewing associated ESCC and OSCC cases which can help us to design our future course of research in the field of cancer genetics as well epigenetics.