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 and esophageal cancer. 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 (betel nut) with Piper betle leaf 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 cancer. 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 inadequately evaluated. 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 people in Meghalaya chew one quarter of the raw betel nut (RBN), which is wet and unprocessed, along with a portion of the betel leaf with lime smeared on it. 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 RBN chew during the course of the day. East Khasi Hills, a district in the Meghalaya state 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 revealed 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. Betel nut chewing with or without tobacco has been shown to be independently associated with the development of esophageal cancer in Assam, India. 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.

Furthermore, LOH could also be observed in neoplastic and apparent phenotypically normal preneoplastic cells that may eventually progress to become cancer. 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. 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. 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. As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns.

Loss of the INK4a/ARF locus results in deregulation of both, p53- and pRB- pathways and hence uncontrolled cell proliferation. Approximately 50% OSCC tumors stain positive for p53. 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. 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. Functional loss of the Rb gene frequently occurs in the carcinogenic processes of many types of cancer. 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. Studies of genetic progression have suggested that p53 alteration occurs at greater frequency in invasive carcinomas than in noninvasive lesions. Several studies have demonstrated that 40–70% of SCCHN contain mutations in exons 5–9 at the p53 locus.

An association between aberrant pRb and p53 expression was observed in bladder and several other types of cancer. 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.

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. p16INK4a is a cyclin-dependent kinase inhibitor functioning upstream Rb. It can negatively regulate cell cycle progression by preventing the phosphorylation (inactivation) of Rb during G1 phase. p14ARF restrains cell growth by abrogating Mdm2 inhibition of the p53 activity, and therefore facilitates p53 mediated cell cycle arrest and apoptosis. It was demonstrated that oncogenic Ras elicits an anti-tumorigenic response mediated by the up-regulation of both p14ARF and p16INK4a, which in turn activate the tumor suppressors p53 and pRb, respectively. p14ARF provides a failsafe mechanism for defective Rb pathway by its inducibility via deregulated E2F-1 activity resulted from Rb
inactivation. Compared with p14ARF and p16INK4a, p15INK4b is less prominent as a tumor suppressor. In contrast to p16INK4a, which is activated by intracellular stimuli, p15INK4b suppresses cell growth in response to extracellular stimuli such as TGF-β. Inactivation of the Rb and p53 tumor suppressor pathways is observed in most human cancers. 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.

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. 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 cancer samples (37 oral and 62 esophageal) 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 (MS-PCR) to detect the promoter hypermethylation of p16INK4a (in 46 samples) and RB1 (in 26 samples) genes.
The results revealed that 22 [59\%] OSCC samples showed LOH for at least one marker locus on 9p and 12 samples did not show LOH for any of the markers. In ESCC, 45 (72\%) samples showed LOH for at least one marker locus on 9p but 11 samples did not have LOH for any marker of 9p. In both type of cancers, a total of 45 tumors showed loss of microsatellite markers D9S1748 and D9S1749 which are located close to exon 1β of CDKN2A/ ARF gene at 9p21 region. It was observed that T4 + T3 showed higher frequency of loss at 9p21 and at 9p22-23 than T2 + T1 which is also found statistically significant (p=0.0438; and p=0.0036).

Chromosome 13q12-14 allelic deletions of at least one marker in 41\% (say 41 cases) of the oral and esophageal samples was observed. A total of 30 (30 \%) tumor samples from both the cancers showed loss of microsatellite markers D13S153 and D13S319 which are located within or proximal to RB1 gene. The age of the patients >50 versus <50 yrs showed statistically significant relation with LOH frequency of the markers on 13q14: D13S218 (p=0.04), D13S153 (P=0.001), D13S233 (P=0.01) in the ESCC samples. It was observed that the involvement of 13q was significantly lower than 9p in both only RBN-chewing associated cancers (p=0.037) and also in cancers induced by both RBN and tobacco (p=0.011). A total of 34 cases showed allelic deletions of at least one marker locus on 17p13.

In oral cancer samples, 13 had loss of 9p, 2 had loss of 17p, 5 demonstrated loss of 9p +13q, 4 had loss of 9p+13q+17p, and 1 had loss of 13q + 17p. A total of 12 samples did not have loss for any markers of any chromosome arm. In case of esophageal cancer samples, 10 had loss of 9p, 2 demonstrated loss of 13q, 1 showed loss of 17p, 10 had
displayed loss of 9p+13q, 10 had shown loss of 9p+17p, 3 had loss of 13+17p and 15 had shown loss of all the three chromosome arms 9p+13q+17p. A total of 11 samples did not have any loss for any markers of present study. The promoter hypermethylation of p16INK4a gene was detected in 30% sample and 11% for RB1 gene. There was a statistically significant difference (p= 0.009) found between the groups of patient with only RBN chewing and RBN + tobacco habit in relation to p16 gene promoter hypermethylation.

We conclude following observations on the basis of present study data:

The Esophagus is the predominant site of cancer development in RBN associated carcinogenesis.

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 with only RBN chewing habit has higher frequency of LOH of 9p23 region along with 9p21 but in RBN + tobacco habit patients, only 9p21 regions had shown higher LOH frequency but not the 9p23 region. This observation suggests 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 the 9p23 region 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.

The 13q14 chromosome arm, which harbour RB1 gene locus, had lower LOH frequency for oral cancer samples. The LOH frequency was predominantly found in esophageal cancer cases. The data suggests that RB1 gene may have a limited role in RBN associated OSCC but it may have role in the progression of RBN induced ESCC.

The 17p13 chromosome arm, which harbour p53 gene, had shown lowest 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 of this study which suggest that these samples may have LOH on other frequently deleted chromosome arms or on the other chromosomal regions of the present study. We need to the study the involvement of other frequently deleted chromosome arms in other cancers such 3p, 8p, 11q etc in RBN induced cancers to better understand the role of genetic alterations like LOH in these cancers.

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. We will have to analyze the promoter hypermethylation of other genes such as RASSF1A, MGMT, DAPK, for them methylation is an important event leading to their inactivation, I RBN associated oral and esophageal cancer.

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