

RESEARCH COMMUNICATION

Distinct Involvement of 9p21-24 and 13q14.1-14.3 Chromosomal Regions in Raw Betel-Nut Induced Esophageal Cancers in the State of Meghalaya, India

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Abstract

Background: Raw betel nut (RBN) chewing is an important contributing factor for esophageal squamous cell carcinoma (ESCC), although associated genomic changes remain unclear. One difficulty in assessing the effects of exclusively RBN induced genetic alterations has been that earlier studies were performed with samples of patients commonly using tobacco and alcohol, in addition to betel-quid. Both CDKN2A (at 9p21) and Rb1 gene (at 13q14.2) are regarded as tumor suppressors involved in the development of ESCC. Therefore, the present study aimed to verify the RBN's ability to induce ESCC and assess the involvement of CDKN2A and Rb1 genes. **Methods:** A panel of dinucleotide polymorphic markers were chosen for loss of heterozygosity studies in 93 samples of which 34 were collected from patients with only RBN-chewing habit. Promoter hypermethylation was also investigated. **Results:** Loss in microsatellite markers D9S1748 and D9S1749, located close to exon 1β of CDKN2A/ARF gene at 9p21, was noted in 40% ESCC samples with the habit of RBN-chewing alone. Involvement of a novel site in the 9p23 region was also observed. Promoter hypermethylation of CDKN2A gene in the samples with the habit of only RBN-chewing alone was significantly higher ($p=0.01$) than Rb1 gene, also from the samples having the habit of use both RBN and tobacco ($p=0.047$). **Conclusions:** The data indicate that the disruption of 9p21 where CDKN2A gene resides, is the most frequent critical genetic event in RBN-associated carcinogenesis. The involvement of 9p23 as well as 13q14.2 could be required in later stages in RBN-mediated carcinogenesis.

Keywords: Loss of heterozygosity - CDKN2A gene - Rb1 gene - promoter hypermethylation - esophageal cancer

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Introduction

Esophageal squamous cell carcinoma (ESCC) is the most common cancers in India and their highest incidence has been reported in north-eastern states of India (Stoner and Gupta, 2001). ESCC age adjusted incidence has been highest in North-Eastern states of India (Chattopadhyay et al., 2010). The morbidity and mortality associated with this disease is a major concern in this region. Of several factors that are implicated for these cancers, consumption of raw betel-nut (RBN; *Areca catechu* L.), instead of dry betel-nut, appears to be highly relevant besides the use of tobacco and alcohol (IARC monograph 1985). In the State of Meghalaya, the variety of Areca nut, locally called 'kwai', is raw, unprocessed with higher contents of alkaloids, polyphenol and tannins as compared to the dried one (Sharan, 1996). The betel-quid used in Meghalaya contains RBN, lime paste and small portion of betel-leaf without tobacco and other constituents. The average age of onset of chewing among tribes is about 12 years and

people swallow the whole quid after chewing instead of spitting it out which could be an important factor for ESCC. 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). Few Indian studies had shown statistically significant association between betel quid without added tobacco and cancer of the esophagus (Jussawalla & Deshpande, 1971; Znaor et al., 2003). The genotoxic potential of arecoline (alkaloid of RBN) in mammalian cells (Deb and Chatterjee 1998: 1999) and the genotoxicity of RBN in RBN heavy-chewers than non-chewers in Meghalaya were demonstrated earlier (Kumpawat and Chatterjee, 2003). The increased frequency of sister chromatid exchanges, chromosome aberrations and micronuclei in exfoliated cells of the buccal mucosa among areca-nut chewers was reported (Dave et al., 1991: 1992), however no attempt has been made to understand the genomic changes induced by RBN leading to cancer.

Inactivation of tumor suppressor genes (TSG) is crucial

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for the development of ESCC. Allelic losses such as homozygous deletion and loss of heterozygosity (LOH) at polymorphic loci are the key events for TSG inactivation. LOH in a cell represents the loss of normal function of one allele of a gene in which the other allele was already inactivated. For TSG with LOH, the unaffected allele could be inactivated either by mutation or by promoter hypermethylation (Fan, 2004). Inactivation of CDKN2A gene, located at 9p21, seems to be an early event in ESCC (Fujiwara et al., 2008) development, resulting in the loss of p16 expression. Genetic alteration of the Rb1-gene, located at chromosome 13q14.2, in esophageal cancers (Contu et al., 2007) has also been reported. Alteration of either pRb1 or p16 may allow cells to enter the S phase of the cell cycle, thereby gaining a growth advantage (Beijersbergen and Bernards, 1996; Serrano et al., 1993).

In the Indian population, a great majority of 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 to verify the ability of RBN to induce ESCC and assess the involvement of p16 and Rb1 gene in the development of ESCC. The present investigation was carried out in 93 ESCC samples of which 34 samples collected from patients with only RBN-chewing habit. Our results indicate that RBN induces esophageal cancer and there is a significant involvement of 9p21 and 9p23 region than 13q14 region. The methylation of CDKN2A gene is considerably higher than Rb1 gene.

Materials and Methods

Patients and tissue samples

A total of 93 esophageal cancer samples, 34 from only RBN chewers and 59 from patients with both RBN and tobacco chewing habit, collected from Nazareth hospital, Shillong, India. This study was approved by the Institutional and Human Ethics Committee. The tissues and corresponding peripheral blood samples were obtained from patients after having their consent for participation and were individually interviewed before taking the biopsy. The age ranged from 27 to 83 years (mean \pm SD; 52.47 \pm 11.17) for esophageal cancer samples collected only from RBN-chewers whereas the age varied from 28 to 81 years (mean \pm SD; 51.11 \pm 9.11) for samples collected from RBN and tobacco users. None of the patients was treated before sampling. Some of the samples collected for this study were from patients with occasional drinking habit. Histologically all the samples were identified as squamous cell carcinoma.

DNA isolation and analysis of LOH

DNA was isolated from tumor and peripheral blood samples. A panel of 9 and 5 dinucleotide polymorphic markers were chosen on the basis of their map position and heterozygosity (Gene Map 99) for a portion of chromosome 9p21-24 and 13q14.1-14.3 respectively. Primers were obtained from Sigma, USA. PCR reaction was carried out in a 10 μ l reaction volume containing

1.5–2.5 mM MgCl₂, 4 pmol of each primer (one-fifth of one of which was end-labeled with [³²P]dATP), 0.2 mM dNTP, 25ng of DNA, and 0.3 unit of AmpliTaq DNA polymerase (Perkin-Elmer Corp., Branchburg, NJ). The amplification was carried out for 30 cycles at annealing temperatures ranging from 52 to 60°C. The PCR products were denatured in sequence stop buffer containing formamide and electrophoresed on a 6% urea-containing polyacrylamide gel, and the dried gels were autoradiographed for 4–16 h. Reduction in signal intensity by more than 50% of one allele in tumor compared with constitutional alleles in the blood by visual examination (and also by using Kodak GelLogic Imaging software) was scored as LOH.

Methylation Specific PCR (MSP)

The methylation pattern of the CpG islands of CDKN2A and Rb1 promoters were determined by bisulfite modification of the genomic DNA followed by methylation-specific PCR. The bisulfite conversion in 2 μ g of extracted tumor DNA was carried out with the EpiTect Bisulfite kit (Qiagen, Sussex, UK) according to the manufacturer's instructions. The genes specific primers for methylated (MF and MR) and unmethylated (UF and UR) used for amplification of methylated and unmethylated promoters of genes CDKN2A and Rb1 are shown below (Simpson et al., 2000; Simpson et al., 2004).

CDKN2A-MF 5'-TTATTAGAGGGTGGGGCGGATCGC-3'
 CDKN2A-MR 5'-GACCCCGAACCGCGACCGTAA-3'
 CDKN2A-UF 5'-TTATTAGAGGGTGGGGTGGATTGT-3'
 CDKN2A-UR 5'-CAACCCCAAACCACAACCATAA-3'
 Rb1- MF 5'-GGGAGTTTCGCGGACGTGAC-3'
 Rb1- MR 5'-CACGTCGAAACACGCCCCG-3'
 Rb1- UF 5'-GGGAGTTTTGTGGATGTGATGTT-3'
 Rb1- UR 5'-CACACATCAAACACACCCCA-3'

Polymerase chain reaction (PCR) was carried out in a total volume of 10 μ l and PCR products were run on 1.5% agarose gels and visualized after ethidium bromide staining.

Statistical Analysis

The association between the categorical variables was assessed using Fisher's exact test. A two-tailed p value of <0.05 was considered as statistically significant.

Results

The present data indicate that the involvement of 9p21-23 is more than 13q14.1-14.3 in the esophageal cancer samples collected from RBN chewers in Meghalaya, India.

Loss of heterozygosity analysis of 9p21-23

The results in Table 1 show the pattern of LOH separately in esophageal cancer samples collected from patients with the habit of RBN-chewing with or without tobacco. The representative gels showing LOH are illustrated in Figure 1A. The analysis revealed deletions in at least one locus in 61.7% and 69.5% of the tumor samples with the habit of RBN-chewing alone or from combined users of RBN and tobacco, respectively (Table 2). The LOH pattern identified three discrete regions of deletion and they are: at proximal region of 9p21 that

Table 1. Frequency of LOH on Chromosome 9p and 13q in Esophageal Squamous Cell Carcinoma Cases of RBN-Chewers with and Without Tobacco.

Band position	Locus	°G	°H	Esophageal Cancer			
				RBN only		RBN + Tobacco	
				°S / °I	LOH (%)	°S / °I	LOH (%)
9p24	D9S1813	8.98	84	33 / 26	6 (23.0)	58 / 46	12 (26.0)
9p23	D9S1849	15.87	83	33 / 27	12 (44.4)	57 / 49	18 (36.7)
	D9S286	17.77	88	34 / 29	8 (27.5)	57 / 49	13 (26.5)
9p22	D9S157	32.20	85	34 / 29	9 (31.0)	57 / 48	17 (35.4)
9p21	D9S1749	36.74	94	34 / 27	11 (40.7)	57 / 57	17 (29.8)
	D9S1748	36.90	92	34 / 32	10 (31.2)	57 / 52	16 (30.7)
	D9S171	45.57	80	34 / 24	6 (25.0)	58 / 40	09(22.5)
	D9S1679	46.25	73	34 / 30	12 (40.6)	56 / 44	19 (43.2)
	D9S169	49.65	84	32 / 23	11 (47.8)	56 / 44	22 (50.0)
13q14.1	D13S218	39.34	68	34 / 28	10 (35.7)	56 / 41	14 (34.1)
13q14.2	D13S1276	41.44	72	34 / 29	10 (34.5)	58 / 36	12 (33.3)
	D13S153	47.50	82	30 / 23	5 (21.7)	57 / 49	20 (40.8)
	D13S319	52.45	82	33 / 26	8 (30.7)	59 / 44	16 (36.4)
13q14.3	D13S233	56.80	75	34 / 30	7 (23.3)	57 / 49	15 (30.6)

°Genetic position, °Heterozygosity, °S-Studied, °I-informative,

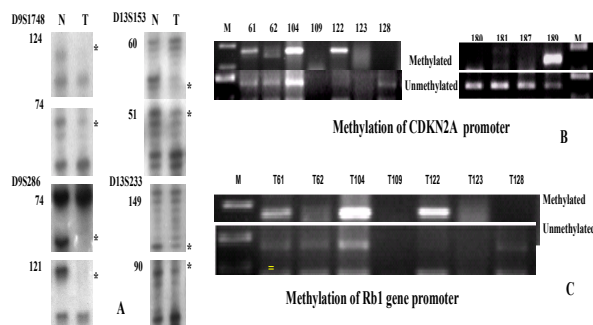


Figure 1 A. Representative Gels Showing Loss of Heterozygosity (LOH) in RBN-Associated esophageal carcinoma in 9p and 13q. N-normal blood DNA; T-tumor DNA; * -indicating LOH. Tumor numbers and markers are shown on left. B. Methylation specific PCR of CDKN2A gene and C. Methylation specific PCR of Rb1 gene. M- marker, T-tumor, each tumor number is mentioned on the top.

spanned by the locus D9S1749 and D9S1748, at distal region of 9p21 where locus D9S169 is present and at 9p23 that spanned by the locus D9S1849 and D9S286. There are 7 samples with the habit of only RBN-chewing and 8 samples with the habit of chewing RBN and tobacco showed loss only at 9p23 region and therefore the loss at 9p23 is independent to other sites at 9p chromosome. The LOH at all of the informative markers showed in 5 esophageal cancer samples out of 34 samples with the habit of only RBN-chewing, suggesting 9p21-23 monosomy. No association was seen between LOH at 9p21-23 region with the age and sex of patients. Loss in microsatellite markers D9S1748 and D9S1749, which are located close to exon 1 β of CDKN2A/ARF gene at 9p21, was noted in 40% ESCC samples with the habit of RBN-chewing alone and in 30% ESCC samples with the habit of combined RBN and tobacco.

Loss of heterozygosity analysis of 13q14.1-14.3

The data indicate lower frequency of LOH in 5 sequence-tagged repeat polymorphism markers mapped to chromosome 13q14. The analysis revealed deletions in

Table 2. Analysis of the Data for Different Parameters in Esophageal Cancer Samples Collected from RBN-Chewers with and Without Tobacco.

	Only RBN	RBN + Tobacco
Number of samples showed no loss or at least one loss in any marker		
9p21-24:		
No Loss	13	18
Loss	21 (61.7%)	41 (69.5%)
Total	34	59
13q14.1-14.3		
No Loss	20	34
Loss	14 (41.2%)	25 (42.4%)
Total	34	59
	p=0.144	p=0.005*
Methylation Status of CDKN2A		
Samples showed methylation (%)	9 (42.8%)	4 (14.3%)
Number of samples Studied	21	28
		p=0.047*
Methylation Status of Rb1		
Samples showed methylation (%)	1 (5.5%)	2 (10.5%)
Number of samples Studied	18	19
	p=0.01*	

Fisher's exact test; * significant

at least one locus in 41.2% and 42.4% of tumors induced by either RBN alone or by combined RBN and tobacco, respectively (Table 2). The LOH at all of the informative markers showed in 7 ESCC samples collected from patients with the habit of only RBN-chewing, suggesting 13q14 monosomy. The LOH pattern did not clearly identify a clear discrete region of deletion in the samples having the habit of RBN-chewing only, however, one discrete region of deletion at 13q14.2 that spanned by the locus D13S153 and D13S319, which are located within or proximal to Rb1 gene, was identified in the samples having the habit of using both RBN and tobacco.

Promoter hypermethylation in esophageal cancers

The methylation status of the promoter region of CDKN2A and Rb1 genes was investigated by methylation-specific PCR (Figure 1 B & C). We utilized the DNA isolated from 49 cancer samples (21 samples from the patients with the habit of RBN-chewing alone) for CDKN2A gene whereas for Rb1 gene, it was monitored in 37 (18 samples from the patients with the habit of RBN-chewing alone) samples only. Promoter hypermethylation in CDKN2A and Rb1 genes was detected in 9 of 21 (42.8%) and 1 of 18 (5.5%) respectively in the samples with the habit of RBN-chewing only (Table 2). Promoter hypermethylation of CDKN2A gene is significantly higher (p=0.01) than the hypermethylation of Rb1 gene in the samples with the habit of only RBN-chewing. From the data it is also clear that hypermethylation of CDKN2A gene was significantly more (p=0.047) in the samples with the habit of RBN-chewing alone than the samples having the habit of using both RBN and tobacco.

Discussion

Present study observed that RBN-chewing alone can induce ESCC and these samples showed higher involvement of 9p21-23 than 13q14 region (p=0.144). Similar observation was also made with the samples having the habit of use both RBN and tobacco (p=0.005). Functional studies have identified that chromosome 9p carries a gene(s) responsible for tumor suppression in ESCC (Fujiwara et al 2008). The present allelotyp

studies identified three putative TSG sites at 9p21 proximal, 9p21 distal and 9p23 site. There is a strong evidence that there could be more than one TSG near 9p21 (including CDKN2A) in multiple human cancers (Illei et al., 2003). The CDKN2A gene, inactivated in a number of human tumors and encodes two functionally unrelated proteins, p16 and ARF/p14ARF (Ruas and Peters, 2003). The primers D9S1748 and D9S1749 are present within the first exon of p14ARF and CDKN2A, respectively. Both CDKN2A and ARF are cell cycle regulators involved in retinoblastoma and p53 pathways of tumor suppression. CDKN2A maintains retinoblastoma in its active, hypophosphorylated, growth-suppressive form by disrupting the CDK4/6-cyclin D complex (Sherr and Roberts, 1995). Deletion of the entire CDKN2A/ARF locus, thus inactivating both pathways, can lead to uncontrolled cell proliferation. The other gene known as methylthioadenosine phosphorylase (MTAP) is also located on 9p21 and is frequently homozygously deleted, along with CDKN2A/ARF, in a wide variety of human tumors and human tumor-derived cell lines (Christopher et al., 2002).

The 9p23 region that spans a 2.5 cM genetic distance is a novel site that showed its involvement particularly in the present ESCC samples. The genes include tyrosine phosphatase delta (PTPRD) and gene amplified in squamous cell carcinoma 1 (GASC1) are present in this region. The biological functions of PTPRD suggest a putative TSG role which is frequently inactivated and mutated in glioblastoma and other human cancers (Veeriah et al., 2009). The presence of these genes in the 9p22-23 region suggests that they may be the targets of deletion in the samples studied by us. Tripathi et al. (2003) identified four discrete areas at chromosome 9 with high LOH in dysplastic and head and neck squamous cell carcinoma (HNSCC) samples from Kolkata. Out of four areas, two sites are within 9p and they are: 9p24-p23 and 9p22-p21. It was suggested that the deletions in 9p21 could be essential for the development of dysplastic lesions of esophageal cancers, whereas the deletions in 9p23-22 region was responsible for progression of the dysplastic lesions to early invasive HNSCC. It is interesting to note that there are tumor samples in this study showed loss only at 9p23 region and therefore the loss at 9p23 is independent to other sites at 9p chromosome. However, it could be that the loss of this site might be contributing for progression of tumors who might have some other loss elsewhere in the genome. In this study, we have observed a frequent loss in the distal region of 9p21 and supporting the view of Xing et al. (1999) who reported that both CDKN2A and p15INK4b genes were frequently inactivated in ESCC. The gene p15INK4b is present at 9p21 distal region and encodes nuclear phosphoprotein, implicated in the negative regulation of the cell cycle mechanisms through the inhibition of CDK4/6-cyclin D complex (Xing et al., 1999).

With regard to chromosome 13q14 the overall frequency of LOH was low and the distinct involvement of 13q14.2, where Rb1 gene is present, is not seen in the present study from the samples collected from RBN-chewers only. Loss of chromosome 13q regions in ESCC

is a frequent event. Gene expression studies by reverse transcription-PCR in microcell hybrids and a panel of ESCC cell lines suggest that the tumor-suppressing effect is not attributed to Rb1, but instead likely involves thrombospondin type I domain-containing 1 (THSD1), a novel candidate TSG mapping to 13q14. Quantitative reverse transcription-PCR detected down-regulation of THSD1 expression in 100% of ESCC and other cancer cell lines (Ko et al., 2008). Therefore, present data indicate the likely involvement of some other TSG(s) including Rb1 gene at 13q14 region in the development of esophageal cancer.

Aberrant promoter hypermethylation is a major epigenetic mechanism for silencing TSGs. Both CDKN2A and Rb1 exhibit frequent hypermethylation in many human cancers (Moreira et al., 2009). In the present study significantly higher ($p=0.01$) number of hypermethylation in CDKN2A gene was observed in the samples with the habit of only RBN-chewing. Moreover, such hypermethylation of CDKN2A gene is significantly lower in the samples of having the habit of use both RBN and tobacco.

However, it is worth mentioning that in the present study none of the esophageal cancer samples collected from the RBN-chewers alone did show loss at only 13q14 region, whereas 13 samples showed loss in 9p23-21 region or methylation of CDKN2A gene without involving 13q14. This indicates that alteration in Rb1 gene occur in later stages of carcinogenesis and could be associated with acquisition of the malignant phenotype as reported earlier (Sony et al., 2004).

Data obtained from 34 RBN-chewers clearly indicate the significant involvement of 9p21 region either by LOH (40.7%) or by promoter hypermethylation (42.8%) of CDKN2A gene. Therefore, it seems that the disruption of CDKN2A gene is an early and most frequent critical genetic event in RBN-associated carcinogenesis. The involvement of 9p23 as well as 13q14.2 could be required at later stages in RBN-mediated carcinogenesis. Further studies may be required to elucidate the role of 9p23 region genes in large cohort of RBN associated precancerous lesions.

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