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Study of oral smear and genetic polymorphism among betel quid chewers and the effect of black tea on oral mucosal cells

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Abstract: *Introduction*: Oral cancer is most common cancer in males and third most common in females; one of the main causative agents is use of chewing betel quid (BQ). Areca nut (*Areca catechu*), a major component of BQ, contains certain alkaloids that give rise to nitrosamines. Mitotic index (MI) , Micronuclei (MN) and CYP2A6 genetic polymorphism were studied among the eastern and north-eastern population. *Methods*: In this present study, subjects were screened from Department of E.N.T. & Oral and Maxillofacial Surgery of RKMSP Hospital, Kolkata, and different areas of Eastern and North-Eastern States of India. Peripheral blood leukocyte cultures were analysed for mitotic index (MI). Polymorphism of *CYP2A6* gene was studied from EDTA blood. Exfoliated cells from the buccal mucosa were examined for micronuclei (MN). *Results*: Some of the cases had more than one addiction. It has been found that micronuclei percentage was higher and mitotic index higher in oral cancer cases than normal. Most of the subjects had betel quid chewing habit. Early metabolizers are susceptible to oral cancer whereas in case of poor metabolizers chances are less. *Conclusion*: Betel quid has an immense role in changing the oral pathology and developing oral cancer.

Keywords: *Oral cancer; Betel quid; Micronuclei; Mitotic index; Tea; CYP2A6 polymorphism*

Introduction

Carcinoma of the oral cavity is one of the 10 most frequent malignant tumours worldwide with major predominance in South-east Asia.[1] Oral squamous cell carcinoma and the most common oral pre-malignancies such as leukoplakia and oral sub mucous fibrosis appear to be related to the habit of betel quid (BQ) chewing in these countries. Areca nut (*Areca catechu*) – a major component of BQ – contains certain alkaloids that give rise to nitrosamines.[2] These BQ-specific nitrosamine may act as an adjunct to tobacco-specific nitrosamines that are strongly implicated as an etiologic factor for leukoplakia and oral submucous fibrosis. Metabolic activation of several nitrosamines was reported to be catalyzed by cytochrome P450 enzymes (CYPs), large multi-gene family enzymes important in phase I metabolic activation reaction.[3] Reactive oxygen species are generated in the oral cavity during BQ chewing due to the addition of slaked lime [$Ca(OH)_2$] into BQ.[4] Genetic defects in *CYP2A6* gene may also effect susceptibility to pre-carcinogen in the environment.

Micronuclei (MN) are small extra nuclei formed in the epithelial cell during metaphase and anaphase stage of cell division.[5] MN have been proposed as a good biomarker to assess cytogenetic damage.[6] Percentage of MN formation has been observed in pre-cancerous lesions of the oral cavity of betel quid chewers. Tea as a national drink has been used in India for many centuries. It was considered as a mental stimulant, mood elevator and also advocated for oral cancer.[7] Consumption of Black Tea for the chemo-preventive action has been shown to exert a protective effect against oral cancer.[8] CYP is a heme-containing enzymes which catalyzes the metabolism of wide variety of exogenous compounds such as drugs, dietary chemicals and environmental pollutants.[9]

Among the xenobiotics metabolizing enzymes, the CYP2A family has characteristics of its catalytic properties to nitrosamines.[10] EM is known as "early metabolizer" and PM as "poor metabolizer". The PM phenotype are incapable of metabolizing the exogenous compound, but EM phenotype are capable of metabolizing the exogenous compound.[11, 12]

Materials and Methods

Screening of Subjects

(I) Camp in Eastern India, (II) Camp in North-East India and (III) Patients attending Maxillofacial and ENT Department of RKMSP Hospital.

(I) Eastern India Camp: Total 220 subjects were screened at a camp held in Bankura, Purba Midnapur, Atghara, West Bengal. Out of whom, 133 were betel quid chewers.

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Table 1: Detailed History of Subjects of Different Areas

Place	No.	Age Group (in Yr)						Addiction					
		Below 30	31–40	41–50	51–60	61–70	Above 70	Smoking	Alcohol	Betel Quid	No BQ Addiction	Tea Drinker	Non Tea Drinker
NORTH EAST CAMP													
(1) Assam, Karimganj	56	1	2	12	24	11	6	9	6	33	23	40	16
EASTERN INDIA CAMP													
(1) Dhulai, Bankura	34	5	20	8	1	0	0	16	14	19	15	34	0
(2) Bibhisanpur, East Midnapur	46	22	13	3	6	2	0	28	29	36	10	40	6
(3) Atghara, North 24 Pgs	89	28	18	21	15	6	1	27	3	56	33	73	16
(4) Narrah, Bankura	51	8	13	12	8	6	4	14	5	22	29	49	2
RKMSP	35	2	7	8	11	7	0	20	8	24	11	29	6
Total	311	66	73	64	65	32	11	114	65	90	121	265	46

Note: Some cases had more than one addiction.

(II) North East Camp: Total 56 subjects were screened at a camp held in Karimganj, Assam. Out of whom, 33 were betel quid chewers.

(III) RKMSP Hospital: Total 2885 cases attending in 1 yr at ENT, OPD and Oral Maxillofacial OPD of RKMSP Hospital had other complications like auditory, nasal, throat and facial problem. Total 35 patient were selected for our study. Total 24 cases were betel quid chewers. Out of 35, 14 cases had pre-cancerous lesion, 13 cases had squamous cell carcinoma, 8 cases had pre cancerous condition.

Methods

Detailed history were taken from all cases by filling up questionnaire. Complete hemogram was obtained by Sysmex KX 21 cell counter.

Leukocyte Culture

Peripheral blood was collected from all cases. Human leucocyte culture was carried out by the method of Moorhead *et al.*,[13] modified by the method of Sharma and Talukder.[14] A total of 4 ml of peripheral venous blood was collected from each donor under aseptic condition with the help of a sterile disposable needle and transferred to a heparinized vial. Leucocyte rich plasma (0.5 ml) was added to a 5 ml of culture media (RPMI 1640, Sigma, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (Sigma) and phytohaemagglutinin M (0.04 ml/ ml of culture media, GIBCO BRL). The cultures were incubated at 37°C. At 70 hr of culture, colchicines (0.2 ml of 0.04%/ml) was added. Two hours later, cells were centrifuged at 1000 rpm for 5 min, treated with pre-warmed KCl (0.075 M) for 15 min, centrifuged at 1000 rpm for 5 min, and fixed in methanol: acetic acid (3:1). Fixed cell suspension was laid on clean grease-free glass slide and air-dried.

The preparation was stained with aqueous Giemsa. All slides were coded, and 1000 blast cells were scored to determine mitotic index per individual.

Micronuclei (MN) Study

The subjects were asked to rinse their mouths with water, and a pre-moistened wooden spatula was used to sample cells from the oral mucosa. The spatula was applied to a pre-cleaned microscope slide. Smears were air-dried and fixed in 80% methanol. Slides were stained by the Giemsa solution, and the MN frequency was scored using the criteria described by Sarto *et al.*[15] The same person scored 1000 cells blindly in each case to determine the MN percentage.

Molecular Study

PCR of different cases were performed with forward and reverse primer for case and control sample at 58°C for annealing temperature with 35 cycle, and total amount of PCR product is 26.5 µl.

Follow-up Study

Black Tea was supplemented to betel quid chewers. Total

Table 2: Total Hemogram of Different Studied Population

Place	No. of Cases	WBC ($\times10^3$)/µl	RBC ($\times10^6$)/µl	Hb (g/dl)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	PLT ($\times10^3$)/µl
I. NORTH EAST CAMP									
(1) Karimganj, Assam	56	6.49±0.20	4.30±0.88	12.24±0.28	37.94±0.76	88.10±0.6	28.39±0.38	31.89±0.33	169.58±9.68
II. EASTERN INDIA CAMP									
(1) Dhulai, Bankura	34	7.86±0.41	4.77±0.1	12.72±0.34	39.51±0.82	83.17±1.46	26.97±0.8	32.13±0.36	147.65±9.76
(2) Bibhisanpur, East Midnapur	46	7.90±0.22	4.81±0.06	13.84±0.19	43.85±0.55	91.46±0.92	28.97±0.4	31.55±0.15	151.69±10.6
(3) Atghara, North 24 Pgs	89	7.49±0.20	4.72±0.06	13.24±0.18	40.88±0.48	86.77±0.77	28.12±0.33	32.33±0.14	154.98±6.36
(4) Narrah, Bankura	51	8.73±0.39	4.85±0.10	13.43±0.31	44.09±0.94	91.14±0.98	27.79±0.44	30.41±0.20	167.45±8.48
III. RKMSP Hospital	35	7.01±0.48	4.65±0.97	12.93±0.33	39.64±1.07	85±1.72	27.3±0.8	32.93±1.06	176.64±14.5

Note: WBC, Total count of WBC; RBC, Total count of RBC; Hb, Haemoglobin; HCT, Hematocrit value; MCV, Mean corpuscular volume; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; PLT, Platelet count. Values are in Mean±SE.

Table 3: Micronuclei and Mitotic Index of Different Studied Population

Place	Micronuclei (%)	Mitotic Index
Dhulai, Bankura	26.29±1.95	3.94±0.23
Bibhisanpur, East Midnapur	12. 31±2.75	8.66±0.67
Atghara, North 24 Pgs	4.76±1.26	5.07±0.60
RKMSP Hospital	[a]	4.54±0.33
Narrah, Bankura	4.61±2.82	4.28±0.62

Values are expressed in Mean±SE.

[a]Study of micronuclei of oral cancer cases was not possible due to severe ulceration and bleeding and cases were unable to open their mouth.

250 g of Black Tea was given to each subject. The subjects were advised to drink three cup of tea brewed with approximately 2.5 g in 100 ml of water. Subjects were asked not to add milk in it or not to boil it for long time. It was advised to keep tea liquor inside the oral cavity for 1–2 min and then drink it. The polyphenol content of the supplied tea was 28±1.86/ 100 g of dry tea. Tea was supplemented to total 117 cases. Follow-up of the cases was done after 6 months.

Results

Overall results of the present study are summarized in Tables 1–5.

Discussion

Oral cancer is one of the leading cancers in most Asian countries.[16] Unstable chromosome aberrations can be studied in epithelial cells by the detection of MN and other nuclear aberrations in exfoliated interphase cells.[17] Casartelli *et al.*[18] observed MN frequencies in exfoliated buccal cells in normal mucosa, precancerous lesions and squamous cell carcinoma. They concluded that the gradual increase in MN counts from normal mucosal to precancerous lesions to carcinoma suggested a link of this biomarker with neoplastic progression. Clinical chemoprevention trials on oral pre-malignancies have used MN in oral mucosa as a surrogate endpoint of cancer.[19] These findings clearly suggest a causal link between MN and cancer. Micronuclei are the small extra nuclei which are

Table 4: Mean Percentage of Micronuclei Before and After Supplementation of Tea

Before supplementation of Tea	After supplementation of Tea
13.86 ±2.70	3.05 ± 0.59

Table 5: Poor Metabolizer and Early Metabolizer of Different Areas

Area	No. of BQ Chewers	Metabolizer	
		Poor (%)	Early (%)
Karimganj, Assam	33	60	40
Dhulai, Bankura	19	16	84
Bibhisanpur, East Midnapur	36	42	58
Atghara, North 24 Pgs	56	90	10
RKMSP Hospital	22	13	87
Narrah, Bankura	24	18	82

Note: Early metabolizer are susceptible to oral cancer whereas in case of poor metabolizers chances are less. BQ, Beetal Quid.

formed in metaphase and anaphase stages. The presence of micronuclei reflects a genotoxic and carcinogenic exposure.[20] Micronuclei have been used as an important marker. The study carried out by Ramirez and Saldanha[21] showed an increase in oral mucosal cell MN frequency in person suffering from cancer and concluded that MN are a product of early events in human carcinogenic process, specially in the oral regions. In our study, it is observed that mean value of mitotic index of all cases having betel quid chewing habit was 5.29 ±0.49 and mean percentage of micronuclei were 12 ± 2.19. In this report, it is shwon that percentage of micronuclei is higher than the normal in cases, and after supplementation of tea micronuclei percentage are lower than before. *CYP2A6* gene deletion reduces oral cancer risk in betel quid chewers in Sri Lanka.[21] Poor metabolizer are less prone to oral cancer than early metabolizer due to *CYP2A6* gene polymorphism. Subjects who have polymorphism in *CYP2A6* are poor metabolizer and showed band in PCR. Early matabolizer had normal *CYP2A6* gene and showed no band in PCR. In our study, it has been found that more than 50% cases from North Eastern states were poor metabolizer, whereas more than 50% cases of Eastern region (except North 24 Pgs) were early metabolizer. Our present work based on case-control study suggest that the *CYP2A6* genetic polymorphism has an impact on susceptibility to oral cancer.

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