Original Article

Association between mitochondrial C-tract alteration and tobacco exposure in oral precancer cases

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ABSTRACT

Introduction: Tobacco exposure is a known risk factor for oral cancer. India is home to oral cancer epidemic chiefly due to the prevalent use of both smoke and smokeless tobacco. To reduce the related morbidity early detection is required. The key to this is detailing molecular events during early precancer stage. Mitochondrion is an important cellular organelle involved in cell metabolism and apoptosis. Mitochondrial dysfunction is thought to be the key event in oncogenesis. Last decade has seen a spurt of reports implicating mitochondrial mutations in oral carcinogenesis. However, there are few reports that study mitochondrial deoxyribonucleic acid (mtDNA) changes in oral precancer. This study aims to understand and link effect of tobacco exposure on mtDNA in oral precancer cases. **Subjects and Methods:** A total of 100 oral precancer cases of which 50 oral leukoplakia and 50 oral submucous fibrosis were recruited in the study and a detailed questionnaire were filled about the tobacco habits. Their tissue and blood samples were collected. Total genomic DNA was isolated from both sources. Mitochondrial C-tract was amplified and bidirectional sequencing was carried out. Mutations were scored over matched blood DNA. **Results:** There was a significant association between the presence of mitochondrial C-tract alteration and duration of tobacco exposure. The probability increased with increasing duration of tobacco consumption. The risk of having this alteration was more in chewers than in smokers. **Conclusions:** Tobacco in both form, chewable and smoke, is oncogenic and causes early changes in mitochondrial genome and chances increases with increasing duration of tobacco consumption.

Key words: D loop, leukoplakia, oral submucous fibrosis, polycytosine tract, tobacco

Introduction

Oral cancer is the 6th most frequent cancer world-wide with more than 90% of it represented by oral squamous cell carcinoma (OSCC) alone. OSCC is often preceded by oral precancer. Today, the term potentially malignant disorder is used in place of oral premalignant lesion and condition that comprises oral precancer.[1] Most common oral precancers occurring in India are leukoplakia and oral submucous fibrosis (OSMF). Malignant transformation rate differs in different type of oral precancers. Long-term studies suggest a malignant transformation rate of leukoplakia around 4-8% while that of OSMF is approximately 7%.[1,2] Depending on the etiology, dietary factors and the level of education and awareness, tumor development and its clinical presentation exhibit sharp contrast when compared

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among different parts of the world. If detected early, mortality related with oral cancer can be reduced by half. Tobacco in both smokeless and smoking forms is a major risk factor for oral cancer. As in most solid tumors, oral epithelial cells undergo genetic alterations in proto-oncogenes and tumor suppressor genes through a multistep process. However, studies detailing effects of tobacco use in oral precancer molecular events are limited.

Tobacco consumption is prevalent in India in various forms such as betel quid consumption, bidi, cigarette smoking, hukka and chelam. Betel quid contains tobacco, tender areca nuts and lime that have been shown to generate reactive oxygen species (ROS) and induce oxidative deoxyribonucleic acid (DNA) damage,[3,4] which can initiate or promote oral carcinogenesis. It is known that tobacco products are very potent free radical generators. In human epithelia cells, tobacco products increase the production of ROS and induce free radical reactions[5] that may be responsible for single strand breaks in DNA[6] especially in the mitochondria where they preferentially accumulate. Given the high susceptibility of mitochondrial deoxyribonucleic acid (mtDNA) to undergo mutations it is probable that such mitochondrial changes are early events in the process of carcinogenesis. Studies on the molecular changes in the mitochondrial genome associated with oral cancer have shown interesting features in both European and Asian cases, like 4977 bp deletion.[7] However, besides ethnicity, there are significant differences between the two populations like the pattern of tobacco consumption and different incidence of oral precancer types like OSMF. This study focuses on the effect of method and duration of tobacco exposure on mitochondrial C-tract alterations in oral precancer cases in Indian population which has been largely ignored.

Subjects and Methods

A hospital based study was conducted at King George's Medical University, (KGMU) Lucknow, India. The study comprised of 100 oral precancer cases of which 50 leukoplakia and 50 OSMF visiting the out-patient clinic in Department of Oral and Maxillofacial Surgery, KGMU for management of oral precancer. The diagnosis of leukoplakia of the oral cavity was confirmed by established clinical guidelines and histopathological examinations. OSMF patients, classified on the basis of following criteria were recruited: decreased mouth opening (<20 mm interincisal distance), palpable fibrous bands in buccal mucosa, blanched oral mucosa (soft palate, buccal, labial, retromolar), reduced elasticity of mucosa, burning sensation of the oral mucosa and restricted tongue movements. Detailed questionnaire was filled and tissue, blood were collected after due consent of the subjects during the routine clinical management of the cases. The blood was taken to serve as self-matched control. Total genomic DNA was extracted from all tissue and blood samples cases at KGMU, Lucknow. Samples were anonymized before processing and approval for the study was obtained from the institutional research ethics committee.

DNA extraction

For tissue sample, DNA was extracted from preselected regions of precancer lesion using 20 mg of formalin fixed tissue. Briefly, tissue sections were washed 6 times with 500 µl 1 × GTE (100 mM glycine; 10 mM Tris-HCL pH 8.0; 1 mM ethylenediaminetetraacetic acid [EDTA]) every 12 h and subsequently put with 300 µl Cell Lysis Solution (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0; 0.5% sodium dodecyl sulphate (SDS)) for 1 h at 55°C. To the solution 10 µl of proteinase K (10 mg/ml) were added every 12 h and kept at 55°C up to 48 h. DNA was subsequently isolated by phenol/chloroform method and re-suspended in 200 ul TE buffer. The whole genomic DNA was extracted from blood using standard phenol chloroform method.

mtDNA C-tract amplification and mtDNA C-tract Sequencing

The mtDNA C-tract amplification was done using a set of primer [Table 1] yielding a 109-bp fragment for all blood and tissue samples. Bidirectional direct capillary was done using BigDye® Direct Cycle Sequencing, Applied Biosystems, USA using manufacturer's recommended protocol. Both Forward and Reverse primers were used to analyze and confirm the sequences in the region of interest. Mitochondrial C-tract sequences from tissue and blood samples of the same individual were compared and any variation in tissue over blood sequence was marked as mutation.

Statistical methods

The main statistical objective in this study was the association of mtDNA mutations with method and duration of tobacco exposure in oral precancer cases. The association of mitochondrial alteration with increasing tobacco exposure was based on a cross-tabulation, fisher's exact test and a logistic regression. All statistical computations were performed using the SAS system version 16 (SAS Institute Inc., USA) and all *P*s reported are two sided.

Table 1: Details of PCR primers and conditions

Primer	Sequence	Amplification conditions
Forward	5′ ACAATTGAATGTCTG CACAGCCACTT 3′	94°C for 5 min, 35 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 45 s, followed by 72°C for 10 min
Reverse	5′ GGCAGAGATGTGTTT AAGTGCTG 3′	

PCR: Polymerase chain reaction

Results

DNA from 100 premalignant lesions and corresponding blood samples with no history of prior or concurrent malignancy was analyzed for mitochondrial C-tract alterations. There were 50 cases of oral leukoplakia and 50 cases of OSMF. Overall, 40 of 100 (40%) patients harbored premalignant lesions that exhibited an additional cytosine at nt position 316 that falls inside Polycytosine tract of mtDNA when compared with uninvolved DNA from corresponding blood sample. Figure 1 demonstrates sequencing chart with additional cytosine in C-tract region at nt 316 position after thymidine residue in mtDNA from lesion while Figure 2 demonstrates normal number of cytosines in corresponding blood sample. Of the 40 samples that had additional cytosine, there were 25 sample from oral leukoplakia and 15 from OSMF [Table 2]. The probability of having additional cytosine in mitochondrial C-tract increased with increasing duration of tobacco exposure, 26.67%, 38.23%, 52.78% with group 1, 2 and 3 respectively [Table 3]. Group 3 (11-30 years) had maximum risk (odds ratio 3.07; $P < 0.05$). The same was true when cases were subdivided according to precancer type, i.e. leukoplakia and OSMF, however the association was not statistically relevant in either case [Figures 3 and 4]. Amongst 100 cases, there were 55 cases which were tobacco chewers whereas 45 were smokers. In each subgroup according to precancer type, there were 29 chewers and 21 smokers in leukoplakia while 26 chewers and 24 smokers in OSMF cases. The probability of having

Table 2: Precancer type details for the presence of additional cytosine

Precancer type	Additional cytosine in tissue samples present or absent		Total
	Present	Absent	
Leukoplakia	25	25	50
Oral submucous fibrosis	15	35	50
Total	40	60	100

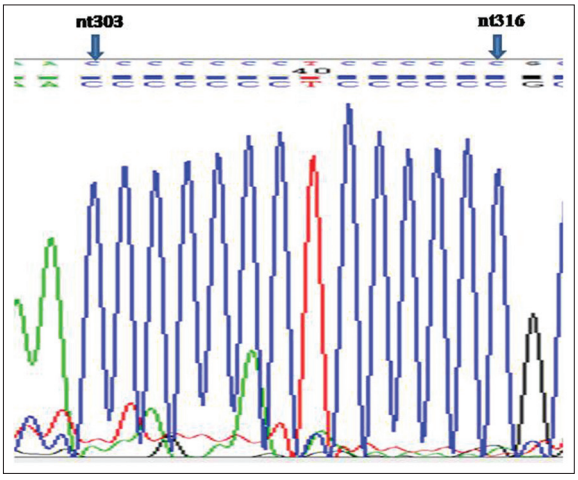

Figure 1: Tissue sample mitochondrial C tract sequence showing 6th (additional cytosine) at nt 316

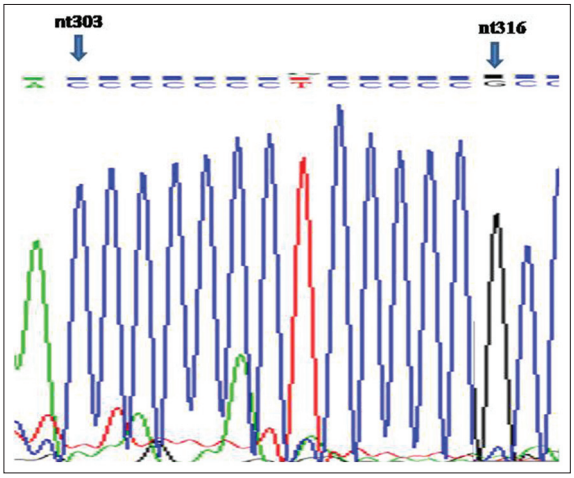

Figure 2: Blood sample mitochondrial C-tract sequence of the same case showing normal cytosine number

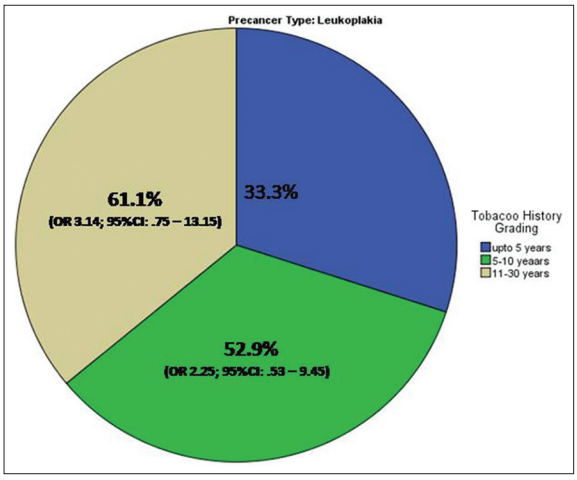

Figure 3: Pie Chart showing the distribution of tobacco history grading in Leukoplakia. (a) odd ratio calculated with reference to group up to 5 years

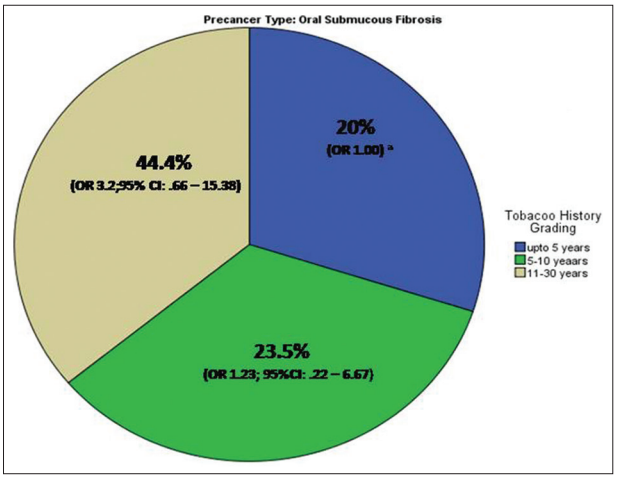

Figure 4: Pie Chart showing the distribution of tobacco history grading in oral submucous fibrosis. (a) odd ratio calculated with reference to group up to 5 years

additional cytosine at nt 316 position inside mitochondria was more in chewers than in smokers, 45.4% and 33.3% respectively. The same was true when the cases were subdivided according to precancer type, 51.7%, 47.6% in leukoplakia and 38.5%, 20.8% in oral submuous fibrosis cases respectively [Table 4]. However, there was no statistically relevant association between method of tobacco consumption and presence of additional cytosine in mtDNA at nt 316 position.

Discussion and Conclusion

A rich and complex body of knowledge gained from cancer research has revealed that cancer is a disease that involves dynamic changes in both the nuclear and mitochondrial genome. Impaired apoptosis is a central characteristic of neoplastic and malignant transformation.[8] The role of mitochondria in energy metabolism, the generation of ROS, aging and the initiation of apoptosis have implicated their importance in tumorigenesis.[9-11] mtDNA mutations in OSCC were first described by Fliss *et al*. in 2000.[12] Although mutations may occur throughout the mitochondrial genome,[13-15] the vast majority of them have been described in the noncoding region of the D-Loop.[16-20] Inside D-Loop there is a highly polymorphic site called C-tract, which is a variable cytosine mononucleotide repeat between nucleotides 303 and 315.

In this study, the presence of C-tract alteration in the form of additional cytosine at nucleotide position 316 was found to be significantly correlated with increasing duration of tobacco exposure. In fact, it is known that 75% of the oral cavity and pharyngeal cancers are attributed to the use of smoked and smokeless tobacco and the rate increases with the amount smoked or chewed and the duration of use.[21] The additional cytosine in our report, we think, is indigenous to Indian population. A study by Alonso *et al*.[22] advanced the hypothesis that a significant ethnic difference in the pattern of DNA mutations may affect susceptibility to environmental factors. This concept is supported by our observation. The diseases selected here is leukoplakia, which has a varied presentation and malignant transformation rate. Another subset, OSMF is indigenous to population from Asia and particularly to Indians. The mutation identified here probably a base transition indicates a possible exposure to mutagens that generate ROS[23] In fact, it is generally accepted that mtDNA mutations are produced during oxidative phosphorylation through mechanisms involving ROS. These mutations may accumulate as the mitochondria lack protective histones and efficient DNA repair mechanisms[24] and is exposed to deleterious ROS generated by oxidative phosphorylation. Tobacco contains several mutagenic ROS-forming substances and carcinogens such as Polycyclic Aliphatic Hydrocarbons, Aza-arenes, Aromatic Amines, Nitrosamines, H_2O_2 and O that can cause DNA damages, especially in mtDNA that has been proved to be more susceptible to oxidative damages than nuclear DNA.[25] In addition, many tobacco smoking metabolic products contain DNA binding agents that can accumulate preferentially in the mitochondria and lead to DNA damage.[26] However, the carcinogenic effects of tobacco occur in the long term of chronic, rather than acute, exposure *in vivo* and therefore ongoing selection pressures may lead to the development of genetic and phenotypic characteristics that could only be studied with certainty in a model of chronic tobacco exposure. This is obvious when we could not find any significant association in intermediate periods of up to 10 years. We think a larger sample size and a more scientific basis for cut-off period are required to overcome such short-comings.

Our data could not differentiate the importance between consuming tobacco either in smoking form or chewing form, though we report higher percentage of additional cytosine amongst chewers in both precancer types.

Table 3: Cross tabulation of tobacco history with C tract alteration

Tobacco history grading (years)	Additional cytosine in tissue samples present or absent		Percentage	OR[a] (95% CI)
	Present	Absent		
Up to 5	8	22	26.67	1.00
6-10	13	21	38.23	1.07 (0.58-4.93)
>10	19	17	52.78	3.07[b] (1.08-8.70)

[a]ORs determined by comparing each category with first group, i.e., up to 5 years,
[b]Statistically significant: $P<0.05$, OR: Odds ratio, CI: Confidence interval

Table 4: Cross-tabulation of method of tobacco consumption with additional cytosine: Pre-cancer type wise

Pre-cancer type	Method of tobacco consumption	Additional cytosine in tissue samples present or absent		Percentage	OR (95% CI)
		Present	Absent		
Leukoplakia	Chewer	15	14	51.7	1.17 (0.38-3.62)
	Smoker	10	11	47.6	1.00
Oral submucous fibrosis	Chewer	10	16	38.5	2.37 (0.67-8.39)
	Smoker	5	19	20.8	1.00
All precancer	Chewer	25	30	45.4	1.67 (0.73-3.76)
	Smoker	15	30	33.3	1.00

ORs determined by comparing each category with smoker, OR: Odds ratio, CI: Confidence interval

Processed tobacco consists of more than 3000 chemical compounds, including at least 30 known carcinogens and cigarette smoke contains around 50 known carcinogens and procarcinogens.[27,28] It is general to presume that since chewing puts tobacco carcinogen in close contact with buccal mucosa, it is more harmful and cancer promoting. Hence, the other side of the story may be, considering smoking to be relatively safe in oral carcinogenesis. However, our data points to the fact that irrespective of the mode of consumption the onset of the carcinogenesis process is the same. This is further proved by the fact that out of seven tobacco-specific nitrosamines that have been identified in tobacco products, two of these, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N'-nitrosonornicotine, are the most important due to their carcinogenic activities and their consistent presence in both unburned tobacco and its smoke, frequently in relatively considerable amounts.[27,28]

Oral cancer is among the most morbid of human cancers and is currently a major global health issue. The high morbidity rate is due to a number of factors including late presentation, failure to respond to treatment regimens currently available and lack of suitable markers for early detection. Progression to OSCC, most common form, is via different stages of oral precancer (mild, moderate, severe) and an intermediate stage (carcinoma *in situ*). Examples of oral precancers include leukoplakia and OSMF. The primary risk factor for oral cancer is tobacco exposure, with a latency period of several decades. Finally, the data presented here point to early genetic changes due to tobacco exposure at an early phase, i.e., an oral precancer. Cataloguing such events may improve morbidity associated with late identification. However, still our efforts are small and in fact recording subtle genetic changes in such population who underwent a transformation from precancer stage to cancer is warranted. This can only be done when a large prospective oral cancer cohort is established and followed-up which cannot be done without multicentric and interdisciplinary approach. This study may provide support for a paradigm in which development of clonal mitochondrial populations with presumed apoptosis resistance are studied with an aim of elucidating tobacco induced carcinogenesis in a chronic exposure model.

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