Micronuclei and chromosomal aberrations in healthy tobacco chewers and controls: A study from Gujarat, India

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SUMMARY

Background: *Tobacco chewing is attributed to oral cancer. Prediction of cancer development by genotoxicity analysis is a major challenge to identify tobacco users at greater risk. Therefore, present study aimed to analyze tobacco related genotoxic effects in chewers monitoring micronuclei (MN) and chromosome aberrations (CA). The biomarkers were compared with non chewer to (i) predict risk for genotoxicity, (ii) estimate synergistic effect of tobacco exposure with level of biomarkers, and (iii) identify best cellular site of measurements for genotoxicity assessment.*

Methods: *Healthy tobacco chewers (n=47); and controls (n=48) were enrolled in the study. The peripheral blood lymphocyte and exfoliated buccal mucosa cells were studied for CA and micro nucleated cell count (MNC) respectively. An arbitrary unit was obtained for Lifetime Tobacco Exposure (LTE) using frequency/day multiplied by duration of years of tobacco use. Data were analyzed using SPSS statistical software.*

Results: *MNC was significantly higher (p=0.001) in chewers than controls. CA was higher in chewers than controls. MNC can differentiate higher tobacco exposure in chewers than CA. Controls having MNC above cutoff level have greater risk of genotoxic exposition (95% C.I.; 1.462-23.26, p=0.012).*

Conclusion: *The present study concludes that MNC is a better surrogate biomarker to predict genotoxicity than CA for tobacco exposure and DNA damage index in tobacco chewers.*

KEY WORDS: *Mouth Neoplasms; Tumor Markers, Biological; Chromosome Aberrations; Micronuclei, Chromosome-Defective; Tobacco Use Disorder*

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Abbreviations:
CA, Chromosome aberrations; LTE, Lifetime Tobacco Exposure; MN, Micronuclei; MNC, Micro nucleated cell count

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INTRODUCTION

There is an increasing effort world-wide to determine the impact of environmental, genetic and life- style factors on genomic stability in human populations. As a result of rapid globalization and changing social attitudes, tobacco and betel quid chewing habits have been increasing worldwide. Tobacco chewing along with various ingredients like areca nut, catechu, lime, cardamom, permitted spices, unspecified flavoring agents have been reported to possess cytotoxic, mutagenic and genotoxic properties (1-3). Tobacco usage in any form is associated with etiology of many diseases for many decades, and any approach aimed at expediting the detection of population sub-groups at increased risk should be considered a high priority task. It may be possible to use genotoxicity assays to identify tobacco users to the DNA-damaging effect over base-line. Many of the substances contained in tobacco are genotoxic and therefore cytogenetic damage seems to be an excellent biomarker for determining the effect of exposure to chromosome-damaging agents in tobacco (1). There have been numerous attempts to establish or even develop tumor markers to determine the susceptibility of normal tissues to transform into cancer. Current predictive indicators can be subdivided into morphologic and molecular. Among molecular predictive indicators, biomarkers of exposure of cytogenetic damage – chromosomal aberrations (CA) and micronuclei (MN) have been used as an important biological endpoint to study population at risk (4). Limitation of DNA damage biomarkers in human studies is the relevance of the accessible tissue in which DNA damage is measured (e.g. erythrocytes, lymphocytes, exfoliated epithelial cells) to the site of cancer studied (e.g. oral, breast, colon). Ideally, measurements are conducted in the target tissue (5). Among cytogenetic markers, MN is studied from exfoliated buccal mucosa of tobacco chewers. MNs are fragments or whole chromosomes, which did not reach spindle poles during mitosis and remained encapsulated at telophase in a separate nucleus. Whereas chromosome aberration (CA) assay detects only the genome damage, MN assay additionally detects chromosome loss or malfunction of mitotic spindle caused by aneugenic mechanisms (6). Therefore in the present study, we aimed to analyze and compare MN in exfoliated buccal mucosa and CA from lymphocyte culture in chewers and controls to identify among these two biomarkers which one is more reliable in terms of (i) risk prediction for genotoxicity, (ii) estimate synergistic effect of tobacco exposure with level of biomarkers, and (iii) identify best cellular site of measurements for genotoxicity assessment.

MATERIALS AND METHODS

Subjects: Healthy tobacco chewers (n=46, males) and healthy non-chewers as controls (n=48 males) were enrolled in the study. Mean age of the subjects in both the groups was 34 years, ranging 18-70 years. Chewers had habit of chewing mixture of tobacco, areca nut and other ingredients like catechu, lime, and unspecified flavoring agents. As tobacco consumption was different in terms of frequency per day and duration of use in years in chewers, the lifetime tobacco exposure (LTE) was calculated. An arbitrary unit obtained using frequency/day multiplied by duration of years was termed as LTE.

Study ethics: The study design and subject consent to participate in the study was ethically approved by hospital based ethical committee of the Institute.

SAMPLES

Blood: Venous blood was collected aseptically in heparinised vials. Peripheral blood lymphocyte cultures were set per sample for CA assay. Per culture 0.5 ml. of whole blood was added to 4.5 ml growth medium (Minimal essential medium with Earle's base and nonessential amino acid containing 20% new born calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin, 3% Phytoheam agglutinin).

Collection of exfoliated buccal mucosa cells: Exfoliated buccal mucosa cells were collected for micro nucleated cell count (MNC) after rinsing the mouth

thoroughly with water. Using a blunt spatula, the cells were scrapped from the oral cavity. The cells were smeared on clean glass slides, fixed with acetic acid: methanol (1:3) air-dried and were stored until staining.

METHODS

Chromosomal Aberrations (CA) Assay: For each subject chromosomal breaks and gaps were analyzed for CA assay. In PBL cultures 2 μg/ml BrdU was added and incubated at 37°C. Cultures were harvested after completion of 48 hrs following 3 hr. treatments with colchicine (0.3 μg/ml). Finally routine hypotonic treatment and fixation protocol was followed up (7). For enumeration of CA frequencies, per sample a minimum of 100 cells in first division were scored from slides stained with 2% Giemsa in Sorenson's buffer (pH 7.0). Identification of CAs was done according to the criteria outlined by WHO (8) and UKEMS (9).

Micronucleus staining: The smears of exfoliated buccal mucosa were stained using the Feulgen plus fast green method with minor modifications. Briefly, the cells hydrolyzed at room temperature with 5N hydrochloric acid for 20 min. Then rinse with distilled water for 90 min. The smear then stained with Schiff's reagent for 2 min, followed by three changes in SO_2 water for 30 min. Smear then washed under running tap water for 30 seconds. The slide then counter-stained with 0.5% fast green in alcohol. A minimum of 1000 cells from each individual was screened for calculating frequency of micro nucleated cells (MNC). The identification of micronucleus was based on the criteria proposed by Sarto et al (10). Evaluation for MN was restricted to oral mucosa cells with both intact nuclei and cytoplasm present. Extra chromosomal cytoplasmic DNA fragments scored as MN were 2-4 μm in diameter and had the same texture and intensity as the nucleus. Fragments scored as MN were in the same focal plane as the nucleus.

Statistical analysis: Data were statistically analyzed using statistical software SPSS (version 15.00). Analysis of variance (ANOVA) test was performed to compare MNC and CA between chewers and controls. Person co-relation analysis was performed to correlate MNC and CA. Odds ratio was calculated using cross-tab analysis for assessment of risk of genotoxicity in controls having MNC and CA above their respective cutoff levels. LTE was used for comparison of tobacco exposure with MNC and CA.

RESULTS

Tobacco consumption and genotoxicity

Figure 1A shows micronuclei and 1B shows CA in a chewer. MNC and CA were compared between chewers and controls using ANOVA test. MNC was significantly higher (p=0.001) in chewers than controls, while CA was higher in chewers than controls but the difference was not statistically significant (Table 1).

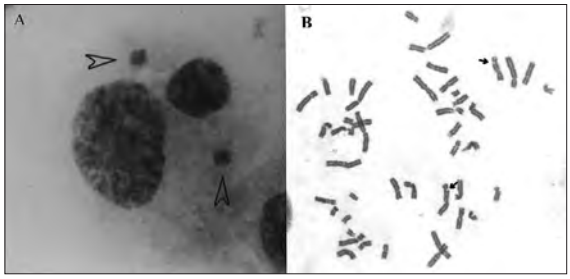

Figure 1. Micronuclei and chromosomal aberrations in a chewer
(A) Micronuclei in buccal cell indicated by arrow. (B) Chromosome aberration break/gap indicated by arrow

Table 1. Comparison of MNC and CA between controls and chewers

	MNC		CA	
	Controls	Chewers	Controls	Chewers
Mean	0.15	0.3239	0.042	0.0502
Standard Error of Mean (S.E.M.)	0.02765	0.04437	0.00408	0.00464
median	0.1	0.3	0.04	0.05
Standard Deviation (S.D.)	0.17045	0.2549	0.02858	0.03179
Range	(0- 0.8)	(0-1.0)	(0-0.17)	(0.01-0.2)
*F value	11.69		1.757	
*P value	**0.001**		0.188	

CA: Chromosomal aberrations; MNC, Micro nucleated cell count
* Analysis of variance (ANOVA)

Correlation between MNC and CA

Table 2 shows Pearson's correlation analysis for MNC and CA in chewers. It is clear that there is significant and positive correlation between MNC and CA in chewers.

Table 2. Correlation analysis between MNC and CA in chewers

Chewers	Pearson Correlation (r value)	Significance (p value)
MNC Vs CA	0.585	**0.0001**

CA: Chromosomal aberrations; MNC: Micro nucleated cell count

Effects of tobacco exposure on extend of DNA damage

As chewers showed higher MNC and CA, we further analyzed whether both biomarkers are associated with tobacco exposure. MNC and CA were compared with percentile value of LTE (Figure 2). It is clear from the graph that after 70 percentile tobacco exposure, MNC is better indicator than CA.

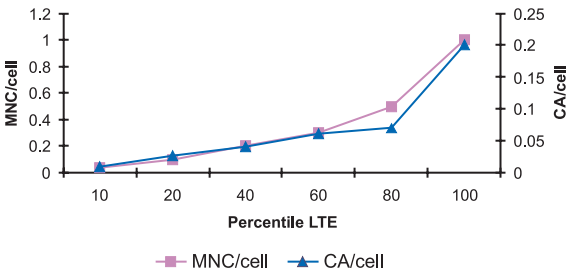

Figure 2. Change in MNC and CA according to LTE in Chewers

Risk prediction in controls for genotoxicity

Odds ratio was calculated using cross-tab analysis for assessment of risk of genotoxicity in controls having MNC and CA above their respective cutoff levels. Cutoff level of MNC and CA was calculated using mean + SD of controls (0.3205 and 0.0706 respectively). It was found that controls having MNC above cutoff level have greater risk of genotoxicity (95% C.I. 1.462 - 23.263; p=0.012, Table 3).

Table 3. Risk assessment for controls showing MNC and CA above cutoff level to predict risk of oral cancer development using odds ratio

Parameter	Ratio	95% C. I.		Significance
		Lower	Upper	
MNC	5.833	1.462	23.263	0.012
CA	0.333	0.033	3.324	NS

CA: Chromosomal aberrations; MNC: Micro nucleated cell count; NS: not significant

DISCUSSION

The use of a biomarker as an indicator of disease development is that the marker will translate into a relationship between exposure and disease (11). The only cytogenetic biomarker that has been outlined previously is the technique of classical metaphase analysis for measurement of CA in human lymphocytes. While MN assay is one of the most commonly used methods for measuring DNA damage in human populations because it is relatively easier to score MN than CA (5).

The present study was designed to test the validity among the two cytogenetic endpoints, MN and CA, as the biomarker of early effect and as a predictive value for a subsequent risk of tobacco related genotoxicity. The rationale for using these biomarkers has been the hypothesis that the extent of genetic damage in peripheral blood lymphocytes reflects similar events in the precursor cells for carcinogenic processes in the target tissues (12) and initiated cells by genotoxic compounds is causally related to cancer risk (13).

It is well known that there is correlation between the CA level in lymphocytes and MN level in exfoliated buccal mucosa cells of persons exposed to environmental mutagens or carcinogens (14,15). In the present study, MNC in exfoliated buccal cells and CA in peripheral blood lymphocyte were positively correlated.

Previously our lab has reported increased MN frequency in areca nut chewers than controls (16). In the present study chewers were heterogeneous in terms of chewing mixture of tobacco, areca nut and other ingredients like catechu, lime and unspecified flavoring agents, MNC was significantly higher in chewers than controls such significant difference was not achieved by CA assay. Stich and Stich observed that saliva of Pan Bahar (a commercially available combination of ingredients like betel nut, catechu, lime, sandal oil, menthol, cardamom, flavor spices, fennel seeds, sugar, waxes, till seeds, colors, etc.) chewers was clastogenic to CHO cells (17). A very high frequency of MN has been observed among tobacco users (18,19). Similarly increase in frequency of MN in "pan masala" consumers has also been reported by Gandhi and Kaur (20).

The association of CA and cancer risk is seen in subjects with known carcinogenic exposure and in those with no history of exposure to carcinogens through occupation or tobacco smoking (21). The results of a report have established diverse buccal cell changes indicated by MN and CA assay and its association with smoking and smokeless tobacco (22). However, we found that CA in chewers was higher but the difference was not statistically significant as seen in MN. MN provides a measure of both chromosome breakage and chromosome loss and as sensitive indicator of chromosome damage as classical metaphase CA analysis (23). The key advantage of the MN assay is relatively easy to score micronuclei as compared to chromosome analysis of CA assay.

We hypothesized that MN and CA would increase as tobacco exposure increases. We found that MNC can better discriminate tobacco exposure index than CA analysis. This may be because MNC is analyzed from target tissue (buccal cell) while CA was analyzed from peripheral blood. MN in buccal cells originate from genome damage events in the basal layer of the oral mucosa while CA analysis from lymphocyte culture allows a measure of genome damage that accumulated while lymphocytes circulate around the body in the quiescent phase. Further lymphocytes have a half-life of 3–6 months and travel throughout the body, integrating genotoxic events across body tissues while in comparison, buccal cells turn over every 21 days (24). Therefore, MNC gives index of recent damage which is tissue specific and reflects the tobacco associated mucosal damage. In the present findings, we found MNC significantly increased in chewers as well as MNC increased as tobacco exposure increases. We have also found that controls without tobacco habits and increased MNC have greater risk of genotoxicity if they are exposed to such agents.

It has been shown frequently that clastogenic and aneugenic effects in somatic cells are associated with the development of cancer (12,13) and a number of earlier studies have shown that the MN assay with exfoliated cells is an appropriate tool to monitor cancer risks in humans caused by exposure to environmental factors or inherited genomic instability (20,22). Therefore, the present findings suggest that chewing habits may cause cancer in the oral cavity or other parts of the upper digestive tract.

It has been hypothesized about direct association between the frequency of MN in target tissues and cancer development, supported by different findings: like, increase in the frequency of MN in target tissues and lymphocytes in cancer patients (6,16). Clinical chemoprevention trials on oral pre-malignancies have used MN in oral mucosa as a surrogate endpoint of cancer (24). A correlation exists between carcinogenicity and genotoxicity for some agents who are able to increase MN frequencies in humans and in animals, e.g., ionizing radiation, ethylene oxide, benzene, tobacco smoke (13). These findings clearly suggest a causal link between MN and cancer.

However, there are aspects of metabolism and susceptibility previously unknown or poorly understood. The biomarkers of exposure and effect and clinical disease can all largely be influenced by susceptibility factors, which include polymorphisms that alter the activity of relevant DNA repair, carcinogen metabolism, and apoptotic pathway genes, as well as dietary factors that alter the activity of such genes (5). In this study some of these factors were nullified as both the study group was from the same socio demographic region with matched age and sex.

In conclusion, The MN test is a simple, practical, inexpensive, and non-invasive screening technique for management of subjects under carcinogenic risks after exposure to genotoxic agents like tobacco. MN test is better indicator for genotoxicity damage than CA. Furthermore, increased micronuclei frequency in the grossly normal appearing oral mucosa of the high risk individuals is associated with greater risk of oral cancer development as suggested by concept of field carcinogenesis. Therefore, genetic composition of micronuclei must be studied to determine if they contain specific genes associated with oral carcinogenesis. Results of such studies could have a significant impact on the future use of micronuclei as a biomarker.

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Conflict of interest

We declare no conflicts of interest.

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