

Comparative Cytogenetic Study of Exfoliative Oral Mucosal cells in Tobacco related Potentially Malignant Disorders in a South Indian Population

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ABSTRACT

Accumulation of genetic alterations induced by the genotoxins by present in betel quid and tobacco leads to oral potentially malignant disorders (PMDs). High levels of malignant transformation of PMDs have been well documented. Micronucleus (MN) assay in exfoliated buccal cells of tobacco related oral PMDs appears to be one of the most suitable tests to assess the toxic effects of various carcinogens in humans. Thus the study was undertaken to observe the cytogenetic damage in the exfoliate buccal cells of tobacco related PMDs and control subjects by MN count. The MN count was analyzed in the exfoliate buccal cells of 15 PMDs patients, of which 8 belonged to leukoplakia (LPK) and 7 to Sub Mucosal Fibrosis (SMF) and compared with the 15 healthy controls. The Mean MN count was scored to estimate the genotoxic damage. The mean and SD values of the MN count of the PMDs were 113.06 ± 17.44 ($3.76\% \pm 0.58\%$) and in controls it was 13.08 ± 5.27 ($0.44\% \pm 0.17\%$). The students 't' test for the MN count between the cases and the controls showed a significant P value (< 0.001). The elevated MN count indicates that the study group belongs to a high risk population for oral cancer. This scientific evidence can be used in support of national campaigns to prevent PMDs from progressing to malignant cancer, by devising intervention strategies and subsequent disease managements to reduce the morbidity and mortality associated with tobacco related oral PMDs.

Keywords : Betel quid, Oral Potentially Malignant Disorders, Exfoliative buccal cells, Genetic damage, Micronucleus

1 INTRODUCTION

The International Agency for Research on Cancer's (IARC), online database, GLOBOCAN 2012[1] has given an estimation of 14.1 million new cancer cases and 8.2 million cancer-related deaths to have occurred in 2012, compared to 12.7 million and 7.6 million, respectively, in 2008. Among the cancers, oral cancer is presently the burning issue in the developing countries. It is the fifteenth commonest cancer in the world and third most common cancer in India. Nearly, 1,30,000 Indians die due to tobacco related oral cancer. Oral cancer is mainly attributed to the use of chewing tobacco since, Indians chew tobacco than smoke it, due to which 75,000 to 80,000 new oral cancer cases have been identified in 2012 and these proportions will increase further by 2025.[1,2] Detection, histopathological investigation, genetic tests, creating awareness for tobacco cessation and treating tobacco

related oral cancer patients especially in their premalignant state are the only hope in reducing the burden of this disease.

Oral cancer arises through an accumulation of genetic alterations, including chromosomal alterations, DNA changes and / or epigenetic alterations. Thus a simple yet a sensitive and specific test for early diagnosis seem to be the need of the hour. The last four decades have witnessed the introduction of a number of relatively rapid genetic tests for detecting the activity of mutagenic and/or carcinogenic chemicals. Among them, Micronucleus (MN) test in exfoliated buccal cells of tobacco related oral premalignant cancer patients appears to be one of the most suitable tests to assess the toxic effects of various carcinogens.[3] Micronuclei are formed by lagging of acentric

chromatid, chromosome fragments or even a whole chromosome that fail to be included in the main nucleus and thus forming one or several secondary nuclei during the cell division. Salama et al.[4] reported, “exfoliated oral cells are excellent for use in monitoring populations exposed to carcinogenic agents because these cells are in direct contact with pollutants that are ingested”. The efficiency of this test for this purpose has been well documented in many studies[5-7] and has been proven to be a reliable biomarker for oral cancer risk.[8-10] The frequency of malignant alterations in oral leukoplakia has ranged from 15.6 to 39.2% in several studies and in India, the rate of malignant transformation ranges from 0.13% to 2.2% per year.[11-13] There also seem to be a positive association between incidence of oral leukoplakia, a premalignant lesion and oral sub mucosal fibrosis, a premalignant condition, with a frequency of malignant change being reported from 3% to 6% [11] and in another report from 0.5 to 6% [14]. As per WHO workshop of 2005, the general term “potentially malignant disorders” (PMDs) is used instead of precancerous lesions & precancerous conditions.

The present micronucleus test to study the chromosomal alteration in the exfoliated cells from the buccal mucosa of betel quid chewers and/or smokers related PMD cases and their equally age- and sex- matched control subjects was conducted to verify the genotoxic effects of the betel quid and tobacco. The main objective of the study was to utilize the findings for early diagnosis and prevention of the disease, paving a way to reduce the mortality associated with the disease.

2 MATERIALS AND METHODS

2.1 Sample Collection and Preparation

This observational study included patients who visited a tertiary care hospital, Mahatma Gandhi Medical College and Research Institute, Pondicherry, India during the years 2012 to 2013. The study group consisted of 15 patients out of which 13 males and 2 females suspected of having premalignant oral cancer on clinical criteria and later histopathologically confirmed as PMDs by the pathologists were included in the study. PMD patients undergoing radiation treatment and patients with chromosomal anomalies like Klinefelter, Turner Syndromes etc were excluded from the study. The control group consisted of equally age- and

sex-matched, who were not habituated to any form of tobacco consumption, pan chewing, smoking or consumed alcohol. Out of the 15 premalignant cancer cases, 8 had leukoplakia (LKP) and 7 sub mucosal fibrosis (SMF). Their age ranged from 23 to 65 years. The site of the oral cancer was also noted. 13 patients suffered PMD on their buccal mucosa region, 3 from tongue region. The patients were characterized into three groups based on their risk factors. Seven patients consumed only betel quid, five patients chewed betel quid and smoked, while three patients smoked and consumed alcohol. The ‘betel quid’ ingredients in the study group consisted of betel leaf, areca nut and slaked lime, and sun-dried tobacco. The duration of their habits was 5-40 years. Before sampling, each individual rinsed his/her mouth thoroughly with tap water. Oral exfoliated cells were scraped from buccal mucosa of control and study group with a moistened wooden spatula. The scraped cells were placed onto pre-cleaned slides. Six slides were made from each subject. The slides were wet fixed and stained with Papanicolaou (Pap).

2.2 Cytological Analysis

The slides were randomized and scored by a single observer. The most commonly used zig-zag method, was followed for screening of slides. Three thousand cells with intact nuclei and cell boundaries were counted for each subject. Cells were examined under the 400X magnification and when MN cells were located, they were examined under the 1000X magnification.

The criterion which was developed by Tolbert et al.[15] was used for counting the micronuclei. The suggested criteria for identifying MN are: rounded smooth perimeter suggestive of a membrane, less than one-third diameter of the associated nucleus, but large enough to discern shape and color, staining intensity similar to that of nucleus, texture similar to that of the nucleus, same focal plane as nucleus and absence of overlap with, or bridge to, the nucleus. Only those structures fulfilling the above-mentioned criteria were recorded as micronuclei (Figure 1). Micronucleated cells were counted out of 3000 intact epithelial cells, and they were expressed as mean micronucleus count.

2.3 Statistical Analysis

Statistical Analysis was done using SPSS 16 Version. P values less than 0.05 is taken as significant.

2.4 Ethical issues

Informed written consent was taken from all the 30 participants. The study was designed in accordance with the Helsinki II declaration and approved by the Institutional Human Ethical Committee, Mahatma Gandhi Medical College and Research Institute, Pondicherry, India.

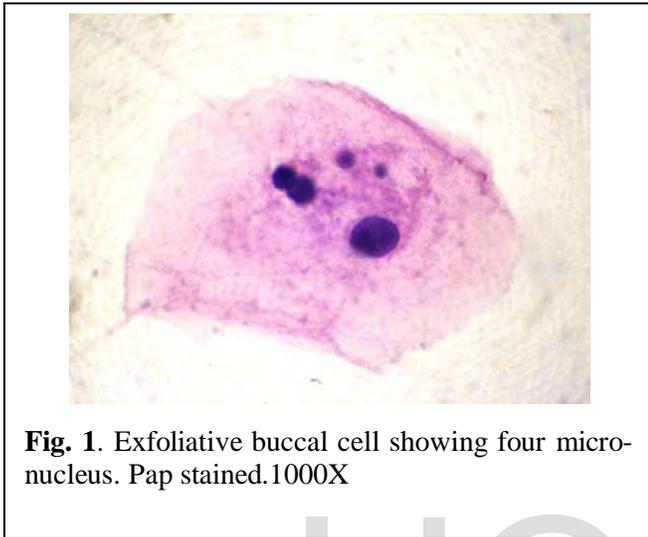


Fig. 1. Exfoliative buccal cell showing four micronucleus. Pap stained.1000X

3 RESULTS

The mean age of the study group was 50.5 ± 13.8 years with 10 patients <50 years and 5 patients ≥ 50 years of age. The mean duration of tobacco related habits was of 22.81 ± 12.07 years of which males had a mean duration of 22.24 ± 12.02 years and females, 21 ± 19.79 years. Students t-test indicated that there was no significant difference of mean micronucleus count between the age ($P=0.736537$), gender ($p=0.537$) and site of the cancer ($P=0.372$). ANOVA for their habits ($P=0.479$) also showed an insignificant P value. There was no correlation (Partial correlation) between the years of habits and their MN count in regard to their different types of habits ($r= -0.184$, $P=0.530$). The mean MN count for LKP was 118.25 ± 19.85 and SMF was 107.14 ± 13.16 . The regression analysis done keeping histopathological impression as a dependable variable, with age, sex, habits, duration of their habits and Micronucleus count as a constant predictors also showed an insignificant P value ($P=0.491$). The Students 't' test for MN count between the two PMDs showed an insignificant P value ($P=0.231$). The 95% confidence interval for LKP ranged between 104.4914

and 132.0086 and for SMF between 97.39514 and 116.8909 [Table 1]. The mean and SD values of the MN count of the controls was 13.08 ± 5.27 ($0.44\% \pm 0.17\%$) and PMDs was 113.06 ± 17.44 ($3.77\% \pm 0.58\%$). The students 't' test for the MN count between the cases and the controls indicated a significant P value (< 0.001) [Table 2].

Table 1: MN count mean values of PMDs

PMDs	N	Mean MN Count	Std. Error of Mean	95% Confidence Interval	
				Lower Bound	Upper Bound
LKP	8	118.250	7.01	104.49	132.00
SMF	7	107.143	4.97	97.395	116.89
Total	15	113.067	4.50	104.24	121.89

PMDs, potentially malignant disorders; LKP, leukoplakia; SMF, submucosal fibrosis; N, total number of individuals; MN, micronucleated cell

Table 2: Students 't' test for MN counts between Cases and Control

Group	N	Mean MN	SD	Std. Error of Mean	t-test	P value
Cases	15	105.40	28.28	7.302	12.43	$<0.001^*$
Control	15	13.06	5.27	1.361		

N, total number of individuals; MN, micronucleated cell; *Significant P value when compared with controls

4 DISCUSSION

MN represent small, additional nuclei formed by the exclusion of chromosome fragments or whole chromosomes lagging at mitosis caused by genotoxins/carcinogens and is considered as an important genetic screening test for analysis of cytogenetic damage in exfoliate buccal cells of tobacco related oral cancer patients. [5,16,17] In India, tobacco related oral cancer is on a high rise and the need control the disease has become a major problem. [18] Worldwide, one of the highest incidence rates of mouth cancer among men is found in Pondicherry (8.9 per 100,000). [19] It is imperative that cost-effective oral cancer screening and awareness initiatives be introduced in high-risk populations like Pondicherry. [20] Thus the micronucleus test in the oral epithelium considered to be one of the important biomarkers was used in the present hospital based study to assess the cytogenetic damage in PMDs

[7,21-24] and utilize the findings to prevent oral PMDs from transforming to malignant cancer.[25,26] The present study showed no association between the age and the occurrence of micronucleus. It is in agreement with many studies,[26,27] but a few authors have shown that the frequency of micronucleus increases with age.[28-30] Although some authors [30,31] have found a significant association in the occurrence of micronucleus and gender, we observed no such association.[26,32,33] Prevalence of PMDs in males in our study is in accordance with many other studies reported in literature.[7,27,34] A few studies have reported nearly 75% of their study group belonging to tobacco related PMDs,[25,34] but in the present study, all the PMDs(100%) had consumed tobacco either in the form of chewing or smoking indicating its utmost usage in the Pondicherry population, substantiating the evidence[19] for the prevalence of world wide highest incidence of oral cancer.

Many Indian states are known for high incidence of tobacco related oral cancer. The site of the cancer varies from state to state. In Gujarat, 43.9% of LKPs occurred on the buccal mucosa and 35.4% at the commissure, whereas in Kerala 64.8% were on the buccal mucosa, 24.3% at the commissures and 6% on the tongue and in Andhra Pradesh, 71.3% were on palate, 8.1% on the commissure, 16.9% on the buccal mucosa and 2.7% on tongue.[35,36] Even though the present study showed that the site of the tobacco related oral cancer showed no association with the mean MN count, we have observed 80 % of PMDs cases having cancer in the buccal mucosa and 20% in the tongue. This could be attributed to the chronic use and the individual habit of the placement of betel quid in a particular site of buccal mucosa.

Our study showed no correlation (Partial correlation) between the years of habits, and the mean MN count which is similar to the findings of Gurjeet and Ajit [37] but Caplashet al.[30] identified duration of the exposure of tobacco showed significant association with the MN frequency.[38,39] The regression analysis done for PMDs with age, sex, habits, duration of their habits and Micronucleus count also showed an insignificant P value indicating the need for a larger sample size. MN studies on PMDs in general,[21,25,40] or individual studies on LKP,[40] SMF,[41] and lichens planus[42] have only so far been

reported. Not many studies have been done comparing different types of PMDs and their MN frequency in a single study group. The present study on MN count in exfoliated cells showed an increased mean MN count in LKP than SMF, similar to the findings of Atul et al.[43] The mean MN count found in the LKP (39.41 ± 6.61) was more than six times than the findings of Khanna et al.[40] (6.15 ± 0.68). Likewise the mean MN% ($3.57\% \pm 0.44\%$) in SMF cases in the present study was twice more than the reporting of Anila et al.[41] ($1.71\% \pm 1.4\%$). Elevated mean MN count in PMDs indicate the study group are genetically susceptible to a more chromosomal damage in their exfoliative buccal cells than other population in India. LKP is more prone to a malignant transformation than SMF. Multiple studies over the years have shown a malignant transformation rate of LKP range 3.6-17.5%, while SMF have shown a malignant transformation rate of about 0.5-6%.[14] This is clearly indicated in the present study while comparing the lower and upper bound mean MN count at 95% confidence interval in LKP and SMF.

The mean MN count noted in PMDs was significantly higher ($P < 0.01$) than the one noted in controls. These results are in accordance with the ones observed in many studies carried on PMDs.[7,21,43,44-46] Our study show a high MN% in healthy controls and in PMDs, which differs from many studies reported so far.[33,47-51] In India, a couple of studies have shown the MN% in controls ranging between 0.15 to 0.35% and in PMDs between 0.5% to 1.9%.[21,42] In the present study, the oral mucosal MN% in the control population was $0.44\% \pm 0.17\%$ and in PMDs, the MN% was $3.76\% \pm 0.58\%$. These observations indicate an elevated cytogenetic damage of the oral epithelium. Similar findings in India of elevated mean MN% cases have been observed by Gosh and Parida.[52] However the population which their subjects were drawn was held to be at a higher risk of oral cancer. A chromosomal study in leukocyte culture of smokers and micronucleus assay in the exfoliative buccal cells of tobacco related oral squamous carcinoma conducted by Uma et al.[53,54] on the Pondicherry population showed a significant elevation of chromosomal damage indicating the population to be at a high risk for cancer. Thus the reason for this high level of MN in our study could be attributed to the fact that Pondicherry belongs to a high risk population for oral cancer.

cer.[18,19] Likewise even though there seem to be no significant association of MN count with that of their types of habits in our study, the betel quid which is a mixture of betel leaf, slaked lime, areca nut and dried tobacco and the combination of smoking with or without alcohol has caused a synergism effect leading to elevated levels of MN% on PMD patients. It is in concordance with the report of many authors.[55-60] The other reason for higher micronucleus count could be due to different staining techniques followed by different authors. Grover et al.[40] has reported a higher MN count with DNA nonspecific Pap stain than DNA-specific Feulgen stain. Even though recently, Dionatas et al.[61] obtained good results using triarilmetano, thiazine and xanthene, getting a good visualization of cell nuclei and micronucleus, the present study followed rapid papanicolaou technique for staining purpose as suggested by many authors [25,27,42,62] since it is very simple to use, less time consuming, and economical.

All the observations and results indicated in the present study clearly show genetic instability[52,63] in exfoliated buccal cells of tobacco related PMDs patients and is in agreement with several recent case-control studies. [21,25,42,46,64-66] The present study, on the need of evaluation of MN in the exfoliate cells of PMD patients also fall in line with the reporting of Kashayps[67], "Simplicity, accuracy, multipotentiality, and large tissue applicability of the MN technology made it attractive in the past and will ensure a key role in the evaluation of mutagenicity and primary prevention in the future".

5 CONCLUSION

Comparing the MN count in the buccal cells of PMDs and the controls, showed a significant higher P value in the PMD patients than the controls. However, the following limitation of this study was noticed. The sample size was less and a definite predictor mean MN range could not be identified. It is thus a necessity that this simple and a reliable MN test on the exfoliate buccal cells of PMDs patient be tested on large samples in the high risk population [18,54] state like Pondicherry.[19] and standardize a predictor MN range for different PMD cases. This scientific evidence can be used in support of national campaigns to prevent tobacco consumption, for devising intervention strate-

gies and subsequent disease managements to reduce the morbidity and mortality from tobacco related PMDs.

CONFLICT OF INTEREST– None

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