Mutagenesis vol. 29 no. 5 pp. 325–334
Advance Access publication 22 July 2014
doi:10.1093/mutage/geu023

Comparative evaluation of genotoxicity by micronucleus assay in the buccal mucosa over comet assay in peripheral blood in oral precancer and cancer patients

Atul Katarkar, Sanjit Mukherjee, Masood H. Khan[1], Jay G. Ray[1] and Keya Chaudhuri*

Molecular and Human Genetics Division, CSIR-Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Kolkata 700032, India and [1]Department of Oral Pathology, Dr R. Ahmed Dental College and Hospital, Kolkata 700014, India

*To whom correspondence should be addressed. Tel: +91 33 2499 5762; Fax: +91 33 2473 5197; Email: keya.chaudhuri@gmail.com

Received on November 21, 2013; revised on April 14, 2014; accepted on April 15, 2014

Early detection and quantification of DNA damage in oral premalignancy or malignancy may help in management of the disease and improve survival rates. The comet assay has been successfully utilised to detect DNA damage in oral premalignant or malignancy. However, due to the invasive nature of collecting blood, it may be painful for many unwilling patients. This study compares the micronucleus (MN) assay in oral buccal mucosa cells with the comet assay in peripheral blood cells in a subset of oral habit-induced precancer and cancer patients. For this, MN assay of exfoliated epithelial cells was compared with comet assay of peripheral blood leucocytes among 260 participants, including those with oral lichen planus (OLP; *n* = 52), leukoplakia (LPK; *n* = 51), oral submucous fibrosis (OSF; *n* = 51), oral squamous cell carcinoma (OSCC; *n* = 54) and normal volunteers (*n* = 52). Among the precancer groups, LPK patients showed significantly higher levels of DNA damage as reflected by both comet tail length (*P* < 0.0001) and micronuclei (MNi) frequency (*P* = 0.0009). The DNA damage pattern in precancer and cancer patients was OLP < OSF < LPK < OSCC, and with respective oral habits, it was multiple habits > cigarette + khaini > cigarette smokers > areca + khaini > areca. There was no significant difference in the comet length and MNi frequency between males and females who had oral chewing habits. An overall significant correlation was observed between MNi frequency and comet tail length with *r* = 0.844 and *P* < 0.0001. Thus, the extent of DNA damage evaluation by the comet assay in peripheral blood cells is perfectly reflected by the MN assay on oral exfoliated epithelial cells, and MNi frequency can be used with the same effectiveness and greater efficiency in early detection of oral premalignant conditions.

Introduction

Worldwide oral cancer ranks eighth position in cancer incidence (1). Oral squamous cell carcinoma (OSCC) constitutes ~96% of all oral malignancies. OSCC is an imperative cause of morbidity and mortality that differ in rate of occurrence and in geological location with estimations of >500 000 new cases per year (2). The aetiology of oral cancer is multifactorial. The established factors that cause oral cancer are mostly smoking, chewing betel quid/tobacco and alcohol intake separately or synergistically (3,4). The commercially available smokeless forms such as khaini (5) or pan (betel quid), gutkha and pan masala consisting of a mixture of lime, areca nut, slaked lime, catechu and other condiments are highly associated with oral cancer (6). Of the malignancies of the oral cavity, 30–80% arise from premalignant lesions such as oral lichen planus (OLP), leukoplakia (LPK) and oral submucous fibrosis (OSF), the range of malignancy transformation rate being 0–5.3%, 0.13–17.5% and 2.3–7.6%, respectively (7,8). Several studies have indicated that the above deleterious habits may induce oxidative stress/reactive oxygen species generation that may lead to DNA damage in form of DNA breaks, double-strand (ds) or single-strand (ss) breaks (9–13). DNA breaks or mis-repaired DNA breaks may lead to a lagging acentric chromosome or chromatid fragments during anaphase from which micronuclei can originate (14). Hence, DNA damage due to oral habits in precancer and malignancy patients may provide a wide array of useful diagnostic and prognostic information along with easy and reliable techniques for their early detection.

The alkaline comet assay (ACA) or single-cell gel electrophoresis is based on the principle that damaged DNA (ds or ss) moves faster than undamaged DNA in an agarose gel. A cell with DNA damage appears in the form of comet, whereas an undamaged cell appears as a halo (15,16). ACA is rapid, versatile and inexpensive, and a small number of cells is required. It is applicable to all eukaryotic cells and sensitive to a smaller amount of DNA damage due to ds, ss and alkali-labile sites. The tail length is directly proportional to DNA damage. The MN assay has emerged as a method for assessing chromosome damage as it facilitates measurement of both chromosome loss and chromosome breakage reliably (17). Micronuclei are characterised by the presence of both a main nucleus and one or more smaller nuclear structures, round/oval in shape, diameter ranges between 1/3 and 1/16 of the main nucleus. There are possibilities of multiple micronuclei depending upon the extent of DNA damage (18). The MN assay is suitable method for DNA damage analysis at differential stages of cancer progression in the response of exposure/accumulation of carcinogen/mutagens as micronuclei start to form much earlier than is observed.

In the last few decades, the comet and MN assays have been widely used tools for evaluation of DNA damage and genomic instability in various forms of cancer (19,20); this has been shown primarily in lymphocytes but has not yet been validated in buccal mucosa cells. Hence, this study mainly aims firstly to evaluate the differential DNA damage induced by habitual usage of processed areca nut products and smoking/smokeless tobacco in differential oral precancer conditions, and secondly, in patients where peripheral collection of blood is not possible, whether the comet assay in peripheral blood lymphocytes can be replaced by the MN assay in oral buccal mucosa cells for monitoring cancer progression.

Materials and methods

Patient selection

Clinically and histopathologically confirmed patients with OLP, LPK, OSF and OSCC were selected from the outpatient department of Dr R. Ahmed Dental College and Hospital in Kolkata, India. A total of 260 consecutive patients with

OLP ($n = 52$), LPK ($n = 51$), OSF ($n = 51$) and OSCC ($n = 54$), together with healthy normal volunteers (controls; $n = 52$) were recruited in order to study the extent of DNA damage using the comet and MN assays. Detailed oral examination was carried out by an oral pathologist. The healthy volunteers without (or <6 months) oral habit with normal oral epithelium were included. The clinically selected and histologically diagnosed patients with OLP, LPK, OSF and OSCC having habitual use of areca nut, khaini or cigarettes in any form were included in the study. Patients suffering from any infectious or contagious disease or with any intractable medical or radiological abnormality, any other white patch-like candidiasis, hypertrophic OLP and lichenoid-like lesions, scleroderma, previous history of surgery, radiotherapy or chemotherapy, or any vitamin or dietary supplement use were excluded from the study. The patients were educated about the study and after obtaining consent a detailed questionnaire of various demographic parameters was recorded. The questionnaire primarily included personal information (age and sex) and asked about risk factors for oral cancer development; habit of chewing pan masala, gutkha, betel quid, areca nut, khaini, cigarette smoking, alcohol ingestion (frequent or occasional), oral hygiene and use of mouthwash. Exposure/duration to deleterious oral habits, number per day of areca nut, khaini and cigarettes used in all habitual users and multiple habits were recorded. The study was duly approved by the human ethics review committee of the institute.

Sample collection

For the comet assay, a peripheral blood sample (2–3 ml) was collected from the antecubital vein, labelled and stored at 4°C with EDTA and protected from light until processed in the laboratory. For the MN assay, individuals were asked to rinse their mouths thoroughly for 2 min with tap water. The exfoliated buccal cells for micronuclei (MNi) analysis were collected from one or both cheeks in the control group, and in the area with lesion in the cases groups, depending on the region where the lesion was located, including cheek, soft and hard palate, dorsal, ventral and lateral surfaces of the oral cavity. The sites of buccal cell collection for smear preparation depended upon the location of the lesion, precancer condition and oral habit. In OLP, sites with white striations, white papules, white plaques, erythema, erosions or blisters affecting predominantly the buccal mucosa were selected. In LPK sites with white or white-and-red lesions that may be either irregular or uniformly flat and thin, nodular or exophytic, wrinkled or corrugated surface with a consistent texture throughout were selected. In OSF sites with tough, leathery texture of mucosa, blanching of mucosa (persistent, white, marble-like appearance which may be localised, diffuse or reticular), quid-induced lesions (fine, white, wavy, parallel lines that do not overlap or criss-cross, are not elevated and radiate from a central erythematous area) were selected. In OSCC site with red or white, painless, non-healing, indurated ulcers were selected along with an unaffected site. The site of the smear was located and wiped with cotton moistened in normal saline to remove slough/surface coatings. In hyperkeratotic lesions such as LPK, surface keratin layer may be scraped off with a curette. Exfoliated oral mucosal cells were spread over glass slides using a spatula. The prepared smears were fixed in the cytology fixative (alcohol and ether in 1:1 ratio) for 30–35 min.

Comet assay

Slides were dipped in 1% normal-melting-point agarose (NMPA) and allowed to dry at 37°C. Once NMPA is solidified, 80 µl of peripheral blood diluted with 80 µl of 1× phosphate-buffered saline was added in a micro-centrifuge tube containing an equal volume (160 µl) of low-melting-point agarose (LMPA, 37°C). A cover slip was placed carefully over the slide so that a uniform layer over the NMPA coat was formed avoiding trapping of air bubbles. The slides were kept over an ice-pack to solidify the gel for 10–15 min. The cover slip was carefully removed and 100 µl of LMPA was added over the gel mixture layer and a fresh cover slip was placed (avoiding trapping of air bubbles), this was also kept over an ice-pack to solidify the gel for 10–15 min. The cover slip was finally removed and the slide was dipped into lysis buffer solution (2.5 M NaCl, 0.1 M Na_2 EDTA and 10 mM Trizma base, 1% Triton X and 10% dimethyl sulphoxide) and refrigerated overnight. After lysis at 4°C, DNA was allowed to unwind under alkaline conditions. The slides were allowed to stay in the cold (4°C) alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 30 min in order to unwind DNA strands and expose the alkali labile sites (alkali unwinding). Electrophoresis was performed for 20 min under alkaline conditions in refrigerator (4°C) at 280 mA and 24 V (~0.74 V/cm). The slides were gently picked up from the alkaline electrophoresis buffer and were placed on a staining tray. The slides were carefully flooded with neutralising buffer (0.4 M Tris, pH 7.5) for 5 min for three times. Following electrophoresis, the slides were neutralised using neutralising buffer (0.4 M Tris, pH 7.5) followed by staining with a fluorescent dye (ethidium bromide 2 mg/ml, Sigma Aldrich). The DNA comet was visualised using a fluorescent microscope (Leica DM 3000, Leica Microsystems). For each sample, two slides were prepared, and the head and tail lengths were measured collectively in 100 cells per sample (50 cells per slide were measured) using Leica QWinPlus digital image processing and analysis software (16,21). The DNA damage was evaluated by the length of the comet tail for each cell.

Micronucleus assay

Fluorescent stain 4′,6′-diamidino-2-phenylindole (DAPI) was used for MNi analysis. DAPI has the highest specificity for DNA and therefore reduces false positives that can arise from hyalokeratin granules (keratin bodies), which may be misinterpreted as micronuclei using a non-specific DNA-stain-like Giemsa stain (22). Also, it gives high fluorescence yield, which is suitable to detect small signals from micronuclei. It also yields the best fluorescence signal for image analysis without cytoplasmic background. Briefly, the slides smeared with buccal cells were washed with 1× PBS for 5 min for three times. The stock solution of DAPI (Invitrogen) was diluted (1 µg/ml) with distilled water and stored at 4°C. Fixed slides were stained for 20 min, rinsed thrice in 1× PBS, air-dried and mounted in the same buffer (22,23). Observations were carried out in a dark room using a fluorescence microscope (Leica DM 3000, Leica Microsystems) equipped with a band-pass filter of 450–490 nm (excitation range: blue). Stained slides were read at ×40 magnification. The criterion for scoring of human buccal micronuclei was followed as per briefly explained by Bolognesi *et al*. (24). MNi identification and structures to be considered were as per the following characteristics: (i) micronuclei are 1/3–1/16 diameter of the main nucleus; (ii) the same plane of focus as the main nucleus; (iii) the same colour, texture and refraction as the main nucleus; (iv) smooth oval or round shape and (v) the nuclear boundary of the micronucleus (MN) should be clearly distinguishable from that of the main nucleus. The buccal cytome model includes biomarkers of (i) DNA damage (micronuclei and nuclear buds); (ii) cell death (condensed chromatin, karyorrhexis, pyknotic and karyolytic cells); (iii) cytokinetic defects or arrest (binucleate cells) and (iv) proliferative activity (basal cell frequency) (24). In this study, DNA damage in buccal cells in terms of micronuclei in differentiated cells (which include both transitional and terminally differentiated cells) with intact nuclei and cytoplasm, non-fragmented, non-clumped and non-overlaid were scored and included in the analysis. The detailed criteria for identification of both transitional and terminally differentiated cells were followed as given by Bolognesi *et al*. (24). Basal cells for MNi analysis were impractical due to low frequency of this type of cell and thus excluded from the study. Nuclear buds, the mechanism behind the formation of which is not known, are thought to arise from the elimination of amplified DNA or from DNA repair. They contain a nucleus with sharp constriction forming a bud, which attaches to main nucleus and has a similar staining intensity to the main nucleus with a diameter a quarter to half that of the nuclear diameter. They were excluded from this analysis because they were observed with very low frequency. A total of 1000 cells per individual were evaluated for the presence of micronuclei. In order to avoid bias, two observers were used to score micronuclei. The measures of DNA damage were evaluated as MNi frequency and expressed as percentages.

Statistical analysis

GraphPad Prism software 5.0 was used for statistical analysis. The data were checked for normality by D'Agostino & Pearson omnibus normality test for age and gender distribution using chi-square test. Non-parametric Kruskal–Wallis test followed by Dunn's post-test was used for comparison of data in studied groups and different oral habits. The one-way analysis of variance (ANOVA) was performed to estimate DNA damage associated with studied groups and differential oral habit. Spearman correlation coefficients were used to calculate the correlation between different oral diagnostic groups. Spearman correlation coefficient was calculated separately to estimate the correlation between MNi frequency and comet tail length (micrometre). Inter-observer variability and inter-individual variability were calculated by using GraphPad Prism. *P*-values of <0.05 were regarded as statistically significant.

Results

Demographic characteristics of the study participants

A representative clinical presentation of control, potentially malignant and cancer patients is described in Figure 1 and their demographic characteristics are summarised in Table I. Significant differences in the mean ages of control (29.34 ± 6.74) with OLP (37.65 ± 11.40), LPK (45.5 ± 15.36) and OSCC (49.18 ± 10.54) were observed with $P < 0.001$ except OSF (31.37 ± 10.16; Table I) by the D'Agostino & Pearson omnibus normality test. Gender distribution was also significantly different in LPK ($P < 0.001$), OSF ($P = 0.024$) and OSCC ($P = 0.02$) females compared with

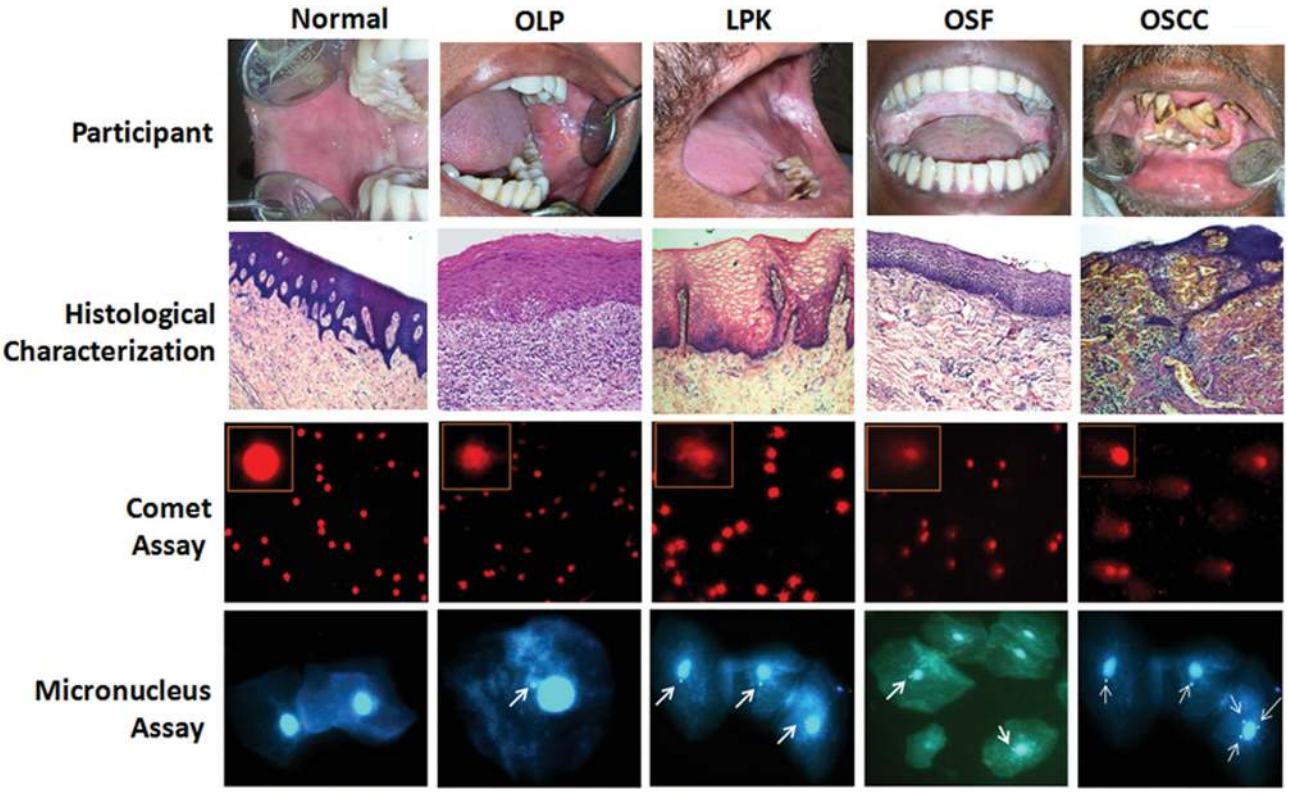

Fig. 1. The pictorial representation of oral precancer conditions (OLP, LPK and OSF) and OSCC, their histological characteristics, comet and micronucleus formation (DNA fragmentation) in comparison to normal.

Table I. Demographic parameters of the control, precancer and cancer participants

Parameter	Normal[a]	OLP[a]	LPK[a]	OSF[a]	OSCC[a]
N = 260	*n* = 52	*n* = 52	*n* = 51	*n* = 51	*n* = 54
Age (year ± SD)	29.34 ± 6.74	37.65 ± 11.40[b]	45.5 ± 15.36[b]	31.37 ± 10.16	49.18 ± 10.54[b]
Sex					
Female	33 (63.46%)	30 (57.69%)	13 (25.49%)	20 (39.21%)	21 (38.89)
Male	19 (36.53%)	22 (42.30%)	38 (74.50%)	31 (60.74%)	33 (61.11%)
P value[c]	–	0.68	<0.001	0.024	0.02
Exposure[d] (year ± SD)	≤ 0.5	2.903 ± 5.35	10.0 ± 6.03	6.54 ± 2.85	12.21 ± 4.86
Habit					
Areca	0 (0.0%)	6 (11.53%)	2 (3.92%)	22 (43.13%)	14 (25.92%)
Areca + khaini	0 (0.0%)	0 (0.0%)	8 (15.68%)	8 (15.68%)	2 (3.70%)
Cigarette	0 (0.0%)	7 (13.46%)	10 (19.60%)	0 (0.0%)	11 (20.37%)
Cigarette + khaini	0 (0.0%)	0 (0.0%)	4 (7.84%)	0 (0.0%)	4 (7.40%)
Multiple habit	0 (0.0%)	2 (3.84%)	25 (49.01%)	21 (41.17%)	19 (35.18%)
No habit	52 (100%)	37 (71.15%)	2 (3.92%)	0 (0.0%)	4 (7.40%)
Pack years[e] ± SD					
Areca	–	171.0 ± 64.14	161.21 ± 89.51	245.82 ± 110.043	170.44 ± 82.11
		8 (3.07%)	14 (5.35%)	51 (5.76%)	34 (13.07%)
Cigarette	–	232.85 ± 90.27	332.30 ± 65.79	240.0 ± 87.31	390.88 ± 199.83
		9 (3.46%)	39 (15.00%)	18 (6.92%)	34 (13.07%)
Khaini	–	–	124.085 ± 14.30	96.63 ± 16.11	101.45 ± 16.63
	–	–	35 (13.46%)	19 (7.30%)	12 (7.30%)
Alcohol[f]	0 (0.0%)	5 (9.80%)	18 (35.24%)	11 (21.56)	19 (35.18%)

[a]The value in parenthesis indicated the percentage of subjects associated with parameter.
[b]Level of significance $P < 0.001$ calculated for distribution of age by using D'Agostino & Pearson omnibus normality test compared with normal in precancer and cancer group.
[c]The distribution of female and male in precancer and cancer patient compared with normal were calculated by chi-square test.
[d]Exposure (year ± SD) indicated the duration of oral habit.
[e]Pack of areca is equal to 10 sachets of gutkha or pan masala or areca containing commercially available products. Pack of cigarette is equal to 10 pieces of cigarette. Pack of khaini equal to 10 split tobacco sachets.
[f]The patient ever/occasional having the habit of alcohol intake.

controls by the chi-square test (Table I). Overall, 55% of the subjects in the study sample were men and 45% were women. Among precancerous and cancerous group, 83.47% had at least one deleterious oral habit, which includes areca (pan masala, gutkha, etc.), areca along with Khaini (chewable tobacco), cigarette smokers only, cigarettes with Khaini and multiple habit such as areca chewing along with cigarettes and Khaini all together. Variations in exposure among the different lesion groups were also observed (Table I). In OLP, most of the patients had no habits (71.15%), while others had a short history of chewing areca (11.53%) or smoking (13.46%). In LPK, ~20% of the patients were smokers, while others mostly had multiple habits (49.01%). In OSF, all subjects were areca chewers (100%) along with chewing Khaini (15.7%). About 36% of patients detected with OSCC had a history of multiple habits, 26% were areca chewers and 20% were cigarette smokers. Alcohol consumption along with other oral habits was found in 35% LPK and OSCC each and in 20% OSF patients (Table I).

Genotoxicity measured by the comet assay in peripheral blood leucocytes

Alkali lysis of DNA followed by electrophoresis revealed a significantly increased ($P < 0.0001$) comet tail length (micrometre) among the OSCC (25.05 ± 4.45 µm) compared with controls (7.079 ± 2.19 µm; Table II, Figure 2A). The mean % tail lengths in different oral diagnostic conditions were allowed for non-parametric Kruskal–Wallis test followed by Dunn's post-test (Figure 2C). Though comet mean tail length was significantly higher (<0.0001) in LPK (16.66 ± 2.23 µm) and OSF (14.13 ± 1.76 µm) compared with controls, it was not as high as in OSCC cases, the most nearest was LPK (Table II, Figure 2C). The OLP cases presented characteristics more like controls (8.154 ± 2.99 µm). The presence of deleterious oral habits worsened the case in OSCC patients (28.45 ± 5.43 µm) (Figure 2C). No significant differences were observed in % tail length compared with male and female in all precancer and cancer groups (supplementary Table 1, available at *Mutagenesis* Online, Figure 2E). When the precancer and cancer group were stratified according to the oral habits, the OLP cigarette smokers showed a significant increase in mean % tail length ($P < 0.0001$) compared with control and in OSF areca with khaini had significant mean % tail length compared with only areca chewers ($P = 0.0033$; Figure 3). LPK and OSCC had similar patterns of DNA damage and multiple habits in all oral lesion groups has highest DNA damage (Figure 3). The order of genotoxicity from precancer to cancer was observed as OLP < OSF < LPK < OSCC by the comet assay. An overall 6.02% inter-observer variability (% $CV^{I\text{-}Ob}$) was observed in the comet assay. The mean % $CV^{I\text{-}Ob}$ with habits, without habit and in different type of oral habits was 6.18%, 6.42% and 8.15%, respectively (Table II). An overall 21.42% inter-individual variability (% $CV^{I\text{-}Id}$) was observed. The mean %$CV^{I\text{-}Id}$ with habits, without habit and in different type of oral habits was 18.11%, 18.54% and 35.40%, respectively (Table II).

Genotoxicity measured by the MN assay in exfoliated oral buccal cells

A progressive increase in MN formation in oral exfoliated buccal cells was noted in precancerous conditions while OSCC presented the highest MNi frequency (15.85 ± 4.39) compared with controls (2.13 ± 1.17; Table II, Figure 2B). The result was consistent with the comet tail length of the respective oral condition. Among the precancerous conditions, LPK presented the highest amount of MNi formation (7.74 ± 3.53; $P = 0.0009$) compared with the other two conditions (OLP = 4.34 ± 2.15; OSF = 6.23 ± 2.68). The mean MNi frequency in different oral diagnostic conditions were allowed for non-parametric Kruskal–Wallis test followed by Dunn's post-test (Table II, Figure 2D).

Table II. Mean ± SD comet length and MNi frequency in oral precancer and cancer according to habit

Attribute	Sample	% Tail length (µm)	$CV^{I\text{-}Ob}$ (%)	$CV^{I\text{-}Id}$ (%)	MNi frequency	$CV^{I\text{-}Ob}$ (%)	$CV^{I\text{-}Id}$ (%)
		Mean ± SD			Mean ± SD		
Normal	52	7.08 ± 2.19	4.30	31.03	2.14 ± 1.17	7.61	54.91
OLP	52	8.15 ± 2.99	4.74	34.20	4.35 ± 2.15	6.72	48.51
LPK	51	16.19 ± 2.86	5.87	11.24	7.75 ± 3.53	6.45	46.12
OSF	51	14.13 ± 1.76	6.77	12.69	6.24 ± 2.68	4.53	32.42
OSCC	54	25.05 ± 4.45	8.43	17.92	15.85 ± 4.39	8.80	20.58
Total	$N = 260$						
With habit							
OLP	15	10.51 ± 2.09	5.73	22.92	5.00 ± 2.76	13.40	51.92
LPK	49	16.66 ± 2.23	6.51	17.77	7.89 ± 3.59	6.49	45.45
OSF	51	14.13 ± 1.76	6.77	12.69	6.24 ± 2.68	4.53	32.42
OSCC	50	28.45 ± 5.43	5.69	19.07	18.08 ± 3.52	2.75	19.48
Total	$N = 165$						
Without habit							
OLP	37	7.19 ± 2.77	6.34	38.59	4.08 ± 1.76	7.13	43.37
LPK	2	12.21 ± 1.42	8.25	11.67	5.50 ± 0.70	11.11	15.71
OSF	0	–	–	–	–	–	–
OSCC	4	18.12 ± 0.97	4.68	5.37	13.50 ± 2.64	9.80	19.60
Total	$N = 43$						
Type of habit							
Areca	44	15.23 ± 4.83	6.25	41.50	8.43 ± 4.00	9.65	64.06
Areca + khaini	18	17.73 ± 6.49	6.19	26.28	11.34 ± 6.24	12.94	54.91
Cigarette	28	20.44 ± 7.17	7.28	38.52	11.35 ± 6.60	12.11	64.11
Cigarette + khaini	8	24.88 ± 7.25	11.45	32.41	12.47 ± 6.42	17.81	50.38
Multiple	67	28.36 ± 7.56	9.60	38.29	16.37 ± 5.42	21.03	49.38
Total	$N = 165$						

$CV^{I\text{-}Ob}$, coefficient of inter-observer variability; $CV^{I\text{-}Id}$, coefficient of inter-individual variability.

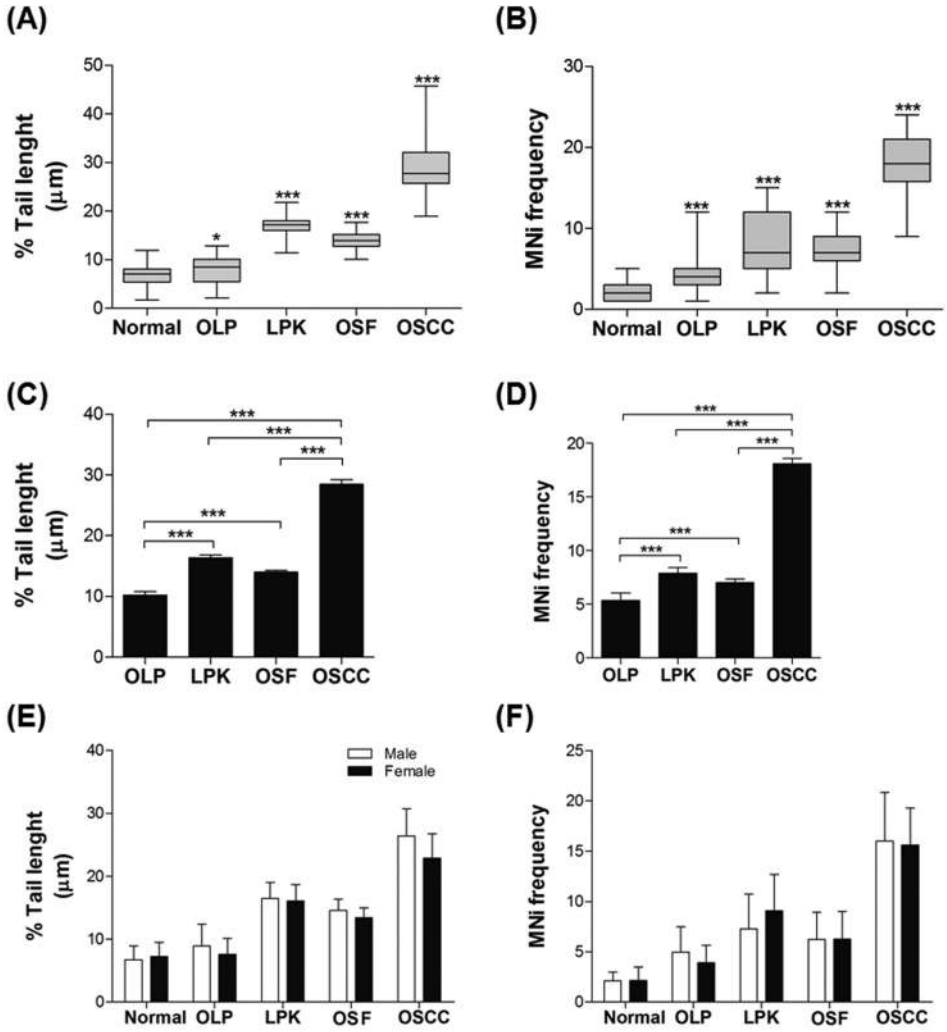

Fig. 2. Comparative DNA damage in different diagnostic conditions analysed by Kruskal–Wallis test fallowed Dunn's post-test (**A**) by ACA, mean ± SD % tail length (**B**) by MN assay, mean ± SD MNi frequency. *, ** and *** represent the level of significance at $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively. The graphical representation of DNA damage with respect to oral habit (pack years) in the different lesion group in precancer and cancer group, (**C**) by comet assay, mean ± SD % tail length (**D**) by MN assay, mean ± SD MNi frequency. (**E**) Graphical representation of mean ± SD % tail length observed in female and male in individual disease group and (**F**) graphical representation of mean ± SD MNi frequency observed in female and male in individual disease group. MNi frequency was calculated as micronuclei per 1000 cells and expressed as percentage (%).

Similar results to those obtained with the comet assay were observed. LPK shows significantly higher mean MNi frequency compared with OLP and OSMF ($P < 0.0001$; Figure 2D). Likewise, no significant differences were also observed in MNi frequency between males and females in all precancer and cancer groups (supplementary Table 1, available at *Mutagenesis* Online; Figure 2F), suggesting that DNA damage was independent of any gender (Figure 2E and F). After stratification of precancer and cancer group according to differential oral habits, results were similar to those obtained with the comet assay. In OLP, a significantly higher MNi frequency ($P < 0.0001$) was observed among the patients who smoked only cigarettes (5.85 ± 3.8) and those who had multiple habits (6.05 ± 0.7). In LPK, MNi frequency was significantly ($P = 0.0296$) correlated among those who smoked only cigarettes (6.10 ± 3.66) and those with multiple habits (9.12 ± 3.50). In OSF, areca chewing was significantly correlated with multiple habits (7.71 ± 1.82) along with areca ($P = 0.0322$; Figure 3). In OSCC, the mean MNi frequency observed is almost equal in all oral habit groups. We observed the multiple micronuclei (>2) in certain patient samples and their MNi frequency was calculated (supplementary Table 2, available at *Mutagenesis* Online). In LPK, 52.94% of patients showed multiple micronuclei and significantly higher MNi frequency was recorded (12.11 ± 2.49) than overall. Moreover, in this study, we did not found any significant difference in MNi frequency between unaffected (13.71 ± 3.56) and affected sites ($P = 0.898$; supplementary Table 3, available at *Mutagenesis* Online). The order of genotoxicity from precancer to cancer was the same as observed with the comet assay. The overall 6.82% $CV^{I\text{-}Ob}$ was observed in MNi analysis. The mean $CV^{I\text{-}Ob}$ with habits, without habit and in different type of oral habits was 6.79%, 9.35% and 14.71%, respectively (Table II). The overall 40.51% $CV^{I\text{-}Id}$ was observed in MNi analysis. The mean $CV^{I\text{-}Id}$ with habits, without habit and in different type of oral habits was 37.32%, 26.23% and 56.57%, respectively (Table II).

Genotoxicity association with exposure of oral habits

The combined description of genotoxicity and exposure of oral habits (in pack years) in different precancer and cancer groups

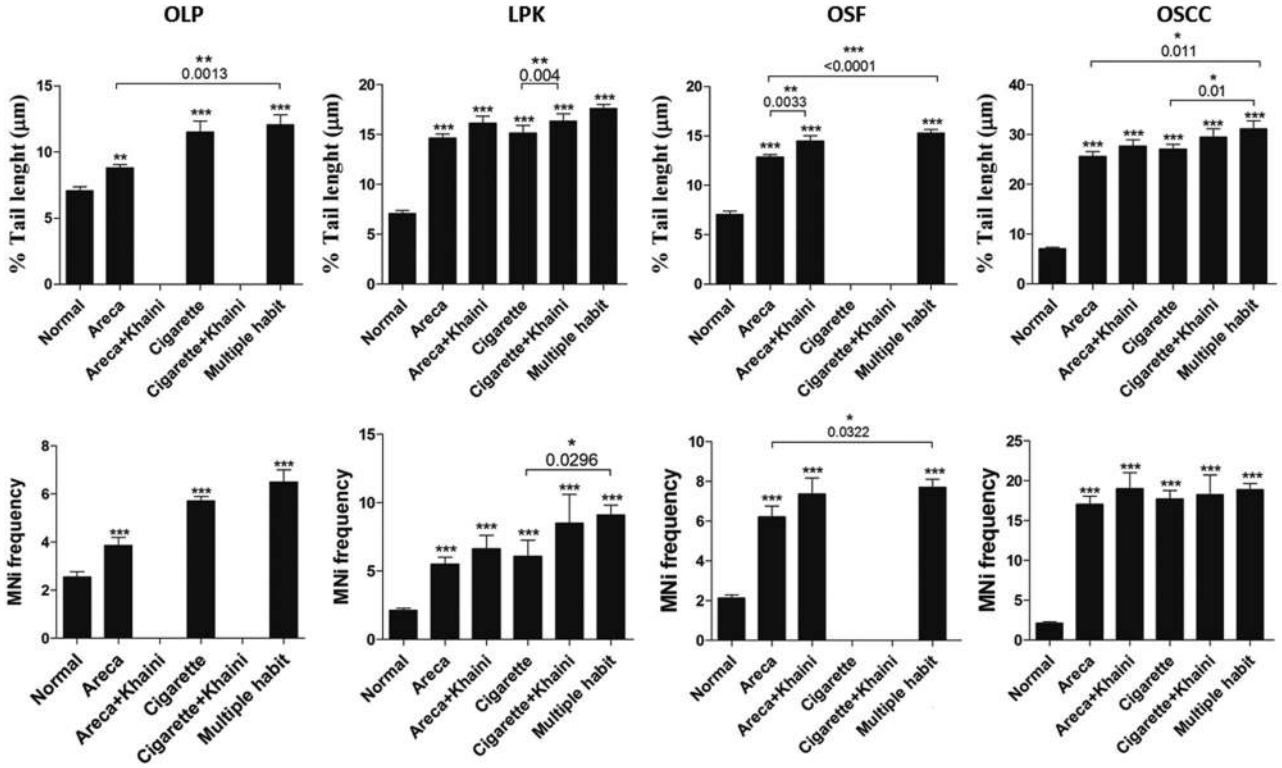

Fig. 3. Comet length and MNi analysis in precancer and cancer condition with different oral habits using Kruskal–Wallis test followed by Dunn's post-test. *, ** and *** represent the level of significance at $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively. MNi frequency was calculated as MNi per 1000 cells and expressed as percentage (%).

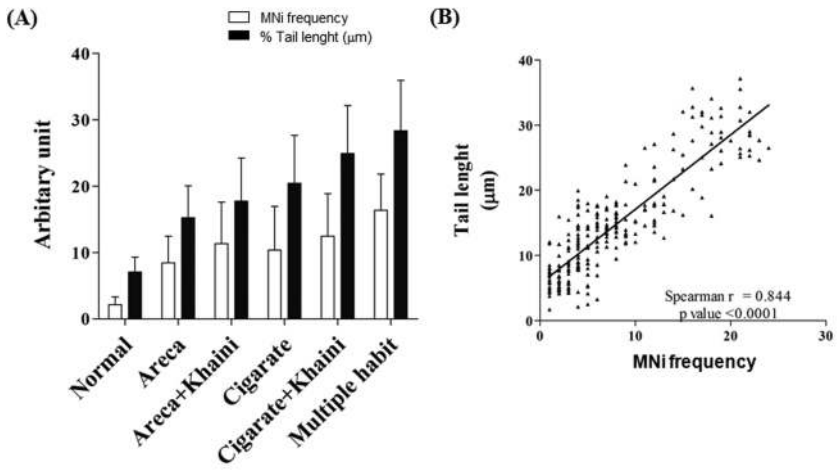

Fig. 4. (**A**) Overall comet length and MNi analysis to estimate DNA damage associated with different oral habits irrespective of oral lesion. (**B**) Spearman correlation between MNi frequency vs. % tail length irrespective of oral lesion and oral habit. MNi frequency was calculated as micronuclei per 1000 cells and expressed as percentage (%).

is shown in Table I. Exposure to areca of 245.82 ± 110.043 pack years was higher in the OSF and exposure to cigarettes of 332.30 ± 65.79 and 390.88 ± 199.83 pack years were higher in LPK and OSCC, respectively. Collectively, one-way ANOVA model for mean % tail length and MNi frequency in between precancer and cancer group with adjusted oral habits show significant variability in the data $R^2 = 0.7919$, $P < 0.0001$ and $R^2 = 0.7260$, $P < 0.0001$, respectively (supplementary Table 4, available at *Mutagenesis* Online). Interestingly, when the study population was stratified according to oral habits, smoking either alone (20.44 ± 7.17 µm) or with khaini (24.88 ± 7.25 µm) posed to be the most debilitating factor in inducing DNA disintegration. This was higher than individuals habituated to a combined habit of areca nut and khaini (17.73 ± 6.49 µm; Table II, Figure 4A). Likely, the same extent of DNA damage was observed in terms of MNi frequency (Table II, Figure 4A). The comet and MN assays showed overall DNA damage patterns, irrespective of oral lesion, were in the order multiple habits > cigarette + khaini > cigarette smokers > areca + khaini > areca (Figure 4A).

Correlation analysis

The Spearman correlation coefficient was calculated in between comet tail length and MNi frequency to ascertain the extent of damage associated either with different oral premalignant and malignant conditions (Table III) or different oral habits (Figure 5). Mean % tail length shows a positive correlation in OSF vs. LPK ($r = 0.41$, $P = 0.002$) and LPK vs. OSCC ($r = 0.33$, $P = 0.015$), whereas a negative correlation was observed in OLP vs. LPK ($r = -0.34$, $P = 0.011$) and OLP vs. OSF ($r = -0.44$, $P = 0.001$). MNi frequency shows a positive correlation in normal vs. OSCC ($r = 0.31$, $P = 0.024$), OSF vs. LPK ($r = 0.47$, $P = 0.0004$) and LPK vs. OSCC ($r = 0.35$, $P = 0.009$), whereas a negative correlation was observed in OLP vs. LPK ($r = -0.36$, $P = 0.009$). When stratified according to different oral habits, areca nut or smoking in association with smokeless tobacco (khaini) emerged as the most genotoxic habit with highly correlated increased tail length and MNi formation ($r = 0.91$ and 0.9, respectively) compared with any other habit either singly or in combination (Figure 5). Moreover, correlation analysis between overall MNi frequency vs. % tail length irrespective of oral lesion and oral habit was highly significant ($r^2 = 0.844$, $P < 0.0001$) suggesting that the MN assay (locally) and the comet assay (systemic) having similar accuracy and precision for detecting DNA damage (Figure 4B).

Table III. Spearman correlation coefficient analyses for DNA damage between oral precancer and cancer group by comet and micronucleus assay

	OLP$^{r(p)}$	LPK$^{r(p)}$	OSF$^{r(p)}$	OSCC$^{r(p)}$
Comet assay				
Normal	−0.022 (0.877)	0.028 (0.839)	−0.113 (0.429)	−0.186 (0.185)
OLP		−0.349 (**0.011**)	−0.44 (**0.001**)	−0.12 (0.396)
LPK			0.414 (**0.002**)	0.335 (**0.0151**)
OSF				0.168 (0.235)
Micronucleus assay				
Normal	−0.19 (0.175)	0.121 (0.397)	0.044 (0.76)	0.311 (**0.024**)
OLP		−0.363 (**0.009**)	−0.159 (0.263)	−0.16 (0.258)
LPK			0.478 (**0.0004**)	0.359 (**0.009**)
OSF				0.171 (0.229)

r(p) represents the Spearman correlation coefficient along with *P* value indicated in parenthesis.
P value <0.05 represent the level of significance and denoted by bold.

Discussion

Cancer progression is a multistep process; genetic alterations induced by DNA damage represent the genetic, epigenetic and phenotypic changes, which are the hallmarks of carcinogenesis. The complex patterns of genetic alteration include premalignant stages to fully developed cancer. It is possible to take advantage of the recent discoveries in specific biomarker development for early detection of cancer progression. These biomonitoring techniques should be cost-effective, easy, rapid, non-invasive, accurate and sensitive. A range of tests can be used for early detection of DNA damage, among which the comet assay is widely practiced (25,26). It has been successfully applied to diagnose DNA damage in screening several cancers like breast cancer (27), lung cancer (28), head and neck cancer (29), bladder cancer (30), thyroid cancer (31), ovarian cancer (32) and prostate cancer (33). However, collection of peripheral blood is invasive and can be unsuitable for anaemic patients. The extent

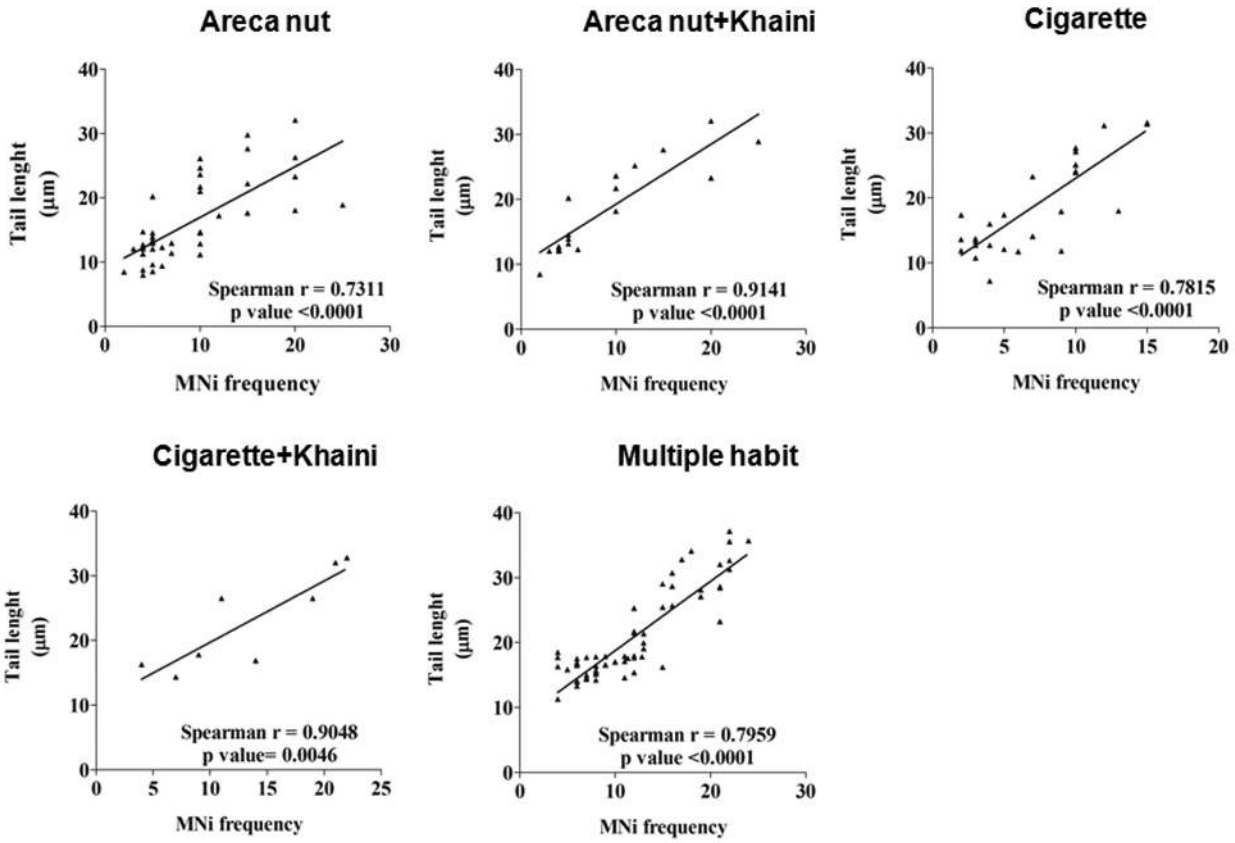

Fig. 5. Spearman correlation between MNi frequency vs. % tail length in differential oral habit. MNi frequency was calculated as micronuclei per 1000 cells and expressed as percentage (%).

of DNA damage in oral premalignancy and malignancy has been successfully ascertained by the comet assay (21,34). Buccal cells form the first barrier to potential carcinogens that can be metabolised to generate potentially reactive products present in pan masala, gutkha, areca nut, betel quid and cigarettes. Nearly 90% of oral cancers are epithelial in origin (18). Hence, oral epithelial cells represent the preferred target site for early genotoxic effects induced by carcinogens. It has been observed that there is a higher comet tail length in buccal epithelium than in leukocytes (35). The comet assay has certain drawbacks when performed in buccal cells. Though lower pH (9.1) was suitable for buccal cells to evaluate DNA damage, it was highly associated with background levels of DNA strand breaks. At this pH, buccal cells are highly resistant to lysis. Even trypsin and proteinase K aided practice caused a high background DNA damage and a homogenous comet head (even fluorescence intensity throughout the nucleoid) (35). Higher pH (>12) aided cell lysis but induced DNA and cellular disintegration, which collectively increased baseline DNA damage and very few cells could be seen in the agarose gel (35–37). The MN assay was used extensively to monitor DNA damage in buccal cells owing to its non-invasive sampling, short turnover time, low DNA repair capacity of the cells and direct correlation with cancer progression, whereas in lymphocytes sampling is invasive, has long turnover time, efficient DNA repair capacity and is indirectly correlated with cancer progression (38,39). In our earlier studies, difficulties were found in collecting peripheral blood samples both from anaemic or trypanophobic patients. The MN assay from the exfoliated buccal cells taken directly from the lesion has certain advantages over the comet assay. Firstly, its collection process is non-invasive, patient friendly and faster. Secondly, the extent of local damage surrounding the lesion (both the affected and the unaffected side) can be ascertained, which helps to determine the spread. And thirdly, the comet assay cannot be performed using exfoliated buccal cells due to its lesser number of collected cells.

MNi staining has been evaluated by varieties of stains in exfoliated buccal cells, among them DNA-specific stains (Feulgen, acridine orange, DAPI and propidium iodide) and non-specific stains (Giemsa, May-Grünwald-Giemsa, Papanicolaou [PAP] and haematoxylin and eosin [H&E]) (40). The results of our study show increases in MNi frequency in smoker compared with control are in contrast with Nerseseyan *et al*. (22) according to which no significant difference in MNi counts were observed between smokers and non-smokers by DAPI staining. The results of Grover *et al*. (41) were though in accordance with our results for increase in MNi count in premalignant conditions, but micronuclei counted by PAP and H&E stains were higher, and so low by Feulgen stain compared with DAPI stain in our case. The results of Jyoti *et al*. (42) stained with acridine orange for OSF are in agreement with our results but show higher MNi counts. Bloching *et al*. (43) showed there was a 2.2-fold increase in the MNi frequency in LPK stains with Giemsa: in contrast, we observed a 3.6-fold increase in overall MNi frequency in LPK. Haldar *et al*. (44) showed a gradual increase in MNi frequency from precancer to cancer compared with normal stained with Giemsa, in agreement with our results and with no significant deviation in MNi frequency/count. In contrast, Fareed *et al*. (45) (MNi stain with Giemsa) and Sellappa *et al*. (46) (MNi stain with May-Grünwald Giemsa) in exfoliated buccal cells of areca chewers found increased MNi frequency, but the MNi count was low and high, respectively, compared with our data. Sanchez-Siles *et al*. (23) observed increased MNi frequency stained with DAPI in OLP compared with normal, and this result and the MNi count are in agreement with our data. Moreover, several studies used PAP stain were positively correlated, in agreement, and with basal MNi frequency/counts not widely different from our results (47–50). These inter-study differences may be due to the exposure to the genotoxic material.

In India, the rate of malignant transformation of LPK is predominant in males particularly in association with chewing tobacco and smoking (51,52). Cigarette smoking is a major risk factor associated with oral LPK (53) and increased MNi formation in exfoliated buccal mucosal cells due to smoking has studied by different groups and has agreement with our results (54,55). A previous report from our lab showed DNA damage measured by the comet assay was greater in LPK associated with smoking (21). As 25% female and 75% male were incident with LPK, among 77% patient has cigarette smoking habit with exposure of 332.3±65.79 pack years observed in our study. Interestingly, a high quantity of multiple micronuclei in single buccal cells was frequently observed in LPK. These observations are in agreement with Mahimkar *et al*. (56), which suggests increased DNA damage in the buccal mucosa of LPK and may be strongly correlated with high incidence with which LPK progresses to malignancy. Collectively in LPK, areca or cigarette smoking and areca + khaini or cigarettes + khaini show relatively similar patterns of DNA damage with the comet assay and with the MN assay. Moreover, MNi frequency was significantly higher with cigarettes + khaini compared with areca, which demonstrates the impact of cigarette smoking in combination with other oral habits.

OSF is a chronic, debilitating disease characterised by progressive fibrosis of the lamina propria and deeper connective tissues, manifest into rigidity and inability to open the mouth (8). OSF is associated with regular use of areca nut chewing, betel nut, gutkha and pan masala (57). Areca nut and arecoline, which is the major contributing factor in the pathogenesis of OSF, have been shown to be cytotoxic and genotoxic to oral epithelial cells *via* DNA damage by oxidative reactive oxygen species generation (9–11) and *N*-nitrosamine formation (5,58,59). In OSF, MNi frequency and % tail length show similar patterns of DNA damage with respect to corresponding oral habits. Recently, several studies have shown increases in MNi in buccal mucosa cells of OSF associated with areca nut and tobacco chewers. Smoking and khaini results in more MNi formation in addition with areca nut and has agreement with our present results (42,47,60).

In India the overall prevalence of OLP, which is a chronic inflammatory condition of unknown origin affecting the lining of mouth with lacy white patches, the malignant transformation rate (7) was 1.5%, relative risk was highest to 13.7% among those who smoked and chewed tobacco. A marked predominance of females was found irrespective of oral habits (61). In this study, 58% female and ~72% subjects with no oral habits were presented with OLP. Increased MNi frequency was observed compared with controls in cigarette smokers than in areca chewers, which may be due to oxidative stress (62). Similar results were observed with the comet assay, but the MN assay was the reliable method for genotoxicity detection in OLP.

The major source of variability in the comet assay arises from the key steps of the protocol, e.g. slide preparation, homogeneity of agarose layer, cell lysis step, electrophoresis condition and scoring (63). Variability in the MN assay in buccal cells arises from differences in scoring by observers, inter-individual variability and type of stain used (64). In this study, inter-observer variability was acceptable in both the comet assay (4.3–8.4%)

and the MN assay (4.5–8.8%). $CV^{I\text{-}Id}$ difference in the comet assay does not vary considerably from premalignant to malignant condition in the subjects with oral habit (17.79–19.07%), but the $CV^{I\text{-}Id}$ difference in the MN assay was more among premalignant to malignant conditions in the subjects with oral habit (43.26–19.48%). A moderately high $CV^{I\text{-}Id}$ (35.4%) was shown by the comet assay, whereas a much higher $CV^{I\text{-}Id}$ (56.57%) was shown by MN assay when observed in types of habit group. This moderate variability for the comet assay in lymphocytes may be due to confounding factors responsible for precancer or cancer reflected in blood due to mutagenic exposure that leads to DNA damage. Subsequent accumulation of DNA damage and the DNA repair capacity of lymphocyte varies with age and sex, which indirectly correlated with disease progression. High $CV^{I\text{-}Id}$ in the MN assay may be due to sensitivity and susceptibility to mutagens to which the buccal cells are directly exposed.

We observed no significant changes in DNA damage in relation to gender; this suggests that DNA damage is independent of gender and reliant on type and duration of exposure to carcinogens and oral habits. The negative correlation of OLP with LPK and OSF may be due to larger difference in the genomic damage accumulated over the time with exposure from oral habits. The significant positive correlation of LPK with OSCC suggests that LPK has the severe DNA damage, which can be directly correlated with its high possibilities to malignancy transformation. The positive correlation of LPK with OSF indicates the quantum of genotoxic insult required to cross the barrier, which leads to neoplasmic transformation.

The major objective of this study was to evaluate DNA damage in buccal cells as a non-invasive sampling, which is of prime importance in early detection of oral cancer. As identification of such abnormalities as cellular proliferation, cell death, DNA damage in the pool of buccal cells is very difficult and only a few studies are documented in the literature, the validation of such a study is quite difficult and may be erroneous. MN identification is very simple, easy and the protocol for MN detection is well standardised. Evaluation of DNA damage extent can be done more accurately by the MN assay by the clinician. However, a more detailed 'cytome-based assay' of oral precancer and cancerous condition will be required for generating more information regarding the biomarkers of cellular proliferation and damage.

Supplementary data

Supplementary Tables 1–4 is available at *Mutagenesis* Online.

Funding

Council of Scientific and Industrial Research (Government of India).

Acknowledgements

A.K. and S.M. are grateful to Indian Council of Medical Research and Council of Scientific and Industrial Research for providing Senior Research fellowship.

Conflict of interest statement: None declared.

References

1. Massano, J., Regateiro, F. S., Januário, G. and Ferreira, A. (2006) Oral squamous cell carcinoma: review of prognostic and predictive factors. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.*, **102**, 67–76.
2. Johnson, N. W., Warnakulasuriya, S., Gupta, P. C., Dimba, E., Chindia, M., Otoh, E. C., Sankaranarayanan, R., Califano, J. and Kowalski, L. (2011) Global oral health inequalities in incidence and outcomes for oral cancer: causes and solutions. *Adv. Dent. Res.*, **23**, 237–246.
3. Boffetta, P., Mashberg, A., Winkelmann, R. and Garfinkel, L. (1992) Carcinogenic effect of tobacco smoking and alcohol drinking on anatomic sites of the oral cavity and oropharynx. *Int. J. Cancer*, **52**, 530–533.
4. Bharali, M. K. and Arangham, G. Synergistic effect of betel quid, tobacco, and alcohol in cigarette smoking induced micronuclei in a population of Arunachal Pradesh, India. *Hum. Ecol. Risk. Assess.*, **20**, 527–536.
5. Stich, H. F., Parida, B. B. and Brunnemann, K. D. (1992) Localized formation of micronuclei in the oral mucosa and tobacco-specific nitrosamines in the saliva of "reverse" smokers, Khaini-tobacco chewers and gudakhu users. *Int. J. Cancer*, **50**, 172–176.
6. Auluck, A., Hislop, G., Poh, C., Zhang, L. and Rosin, M. P. (2009) Areca nut and betel quid chewing among South Asian immigrants to Western countries and its implications for oral cancer screening. *Rural Remote Health*, **9**, 1118.
7. Lončar Brzak, B., Mravak-Stipetić, M., Canjuga, I., Baričević, M., Baličević, D., Sikora, M. and Filipović-Zore, I. (2012) The frequency and malignant transformation rate of oral lichen planus and leukoplakia-a retrospective study. *Coll. Antropol.*, **36**, 773–777.
8. Murti, P. R., Bhonsle, R. B., Pindborg, J. J., Daftary, D. K., Gupta, P. C. and Mehta, F. S. (1985) Malignant transformation rate in oral submucous fibrosis over a 17-year period. *Community Dent. Oral Epidemiol.*, **13**, 340–341.
9. Sundqvist, K. and Grafström, R. C. (1992) Effects of areca nut on growth, differentiation and formation of DNA damage in cultured human buccal epithelial cells. *Int. J. Cancer*, **52**, 305–310.
10. Tsai, Y. S., Lee, K. W., Huang, J. L., Liu, Y. S., Juo, S. H., Kuo, W. R., Chang, J. G., Lin, C. S. and Jong, Y. J. (2008) Arecoline, a major alkaloid of areca nut, inhibits p53, represses DNA repair, and triggers DNA damage response in human epithelial cells. *Toxicology*, **249**, 230–237.
11. Nair, U. J., Floyd, R. A., Nair, J., Bussachini, V., Friesen, M. and Bartsch, H. (1987) Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA in vitro with betel quid ingredients. *Chem. Biol. Interact.*, **63**, 157–169.
12. Yanbaeva, D. G., Dentener, M. A., Creutzberg, E. C., Wesseling, G. and Wouters, E. F. (2007) Systemic effects of smoking. *Chest*, **131**, 1557–1566.
13. Pryor, W. A., Stone, K., Zang, L. Y. and Bermúdez, E. (1998) Fractionation of aqueous cigarette tar extracts: fractions that contain the tar radical cause DNA damage. *Chem. Res. Toxicol.*, **11**, 441–448.
14. Fenech, M., Kirsch-Volders, M., Natarajan, A. T., *et al.* (2011) Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. *Mutagenesis*, **26**, 125–132.
15. Ostling, O. and Johanson, K. J. (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem. Biophys. Res. Commun.*, **123**, 291–298.
16. Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184–191.
17. Fenech, M. (2008) The micronucleus assay determination of chromosomal level DNA damage. *Methods Mol. Biol.*, **410**, 185–216.
18. Thomas, P. and Fenech, M. (2011) Buccal micronucleus cytome assay. *Methods Mol. Biol.*, **682**, 235–248.
19. Samanta, S. and Dey, P. (2012) Micronucleus and its applications. *Diagn. Cytopathol.*, **40**, 84–90.
20. McKenna, D. J., McKeown, S. R. and McKelvey-Martin, V. J. (2008) Potential use of the comet assay in the clinical management of cancer. *Mutagenesis*, **23**, 183–190.
21. Mukherjee, S., Ray, J. G. and Chaudhuri, K. (2011) Evaluation of DNA damage in oral precancerous and squamous cell carcinoma patients by single cell gel electrophoresis. *Indian J. Dent. Res.*, **22**, 735–736.
22. Nersesyan, A., Kundi, M., Atefie, K., Schulte-Hermann, R. and Knasmüller, S. (2006) Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. *Cancer Epidemiol. Biomarkers Prev.*, **15**, 1835–1840.
23. Sanchez-Siles, M., Ros-Llor, I., Camacho-Alonso, F. and Lopez-Jornet, P. (2011) A novel application of the buccal micronucleus cytome assay in oral lichen planus: a pilot study. *Arch. Oral Biol.*, **56**, 1148–1153.
24. Bolognesi, C., Knasmueller, S., Nersesyan, A., Thomas, P. and Fenech, M. (2013) The HUMNxl scoring criteria for different cell types and nuclear anomalies in the buccal micronucleus cytome assay - an update and expanded photogallery. *Mutat. Res.*, **753**, 100–113.
25. Møller, P., Knudsen, L. E., Loft, S. and Wallin, H. (2000) The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging

agents and effect of confounding factors. *Cancer Epidemiol. Biomarkers Prev*., **9**, 1005–1015.
26. Møller, P. (2006) The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. *Basic Clin. Pharmacol. Toxicol*., **98**, 336–345.
27. Rajeswari, N., Ahuja, Y. R., Malini, U., Chandrashekar, S., Balakrishna, N., Rao, K. V. and Khar, A. (2000) Risk assessment in first degree female relatives of breast cancer patients using the alkaline Comet assay. *Carcinogenesis*, **21**, 557–561.
28. Lou, J., He, J., Zheng, W., Jin, L., Chen, Z., Chen, S., Lin, Y. and Xu, S. (2007) Investigating the genetic instability in the peripheral lymphocytes of 36 untreated lung cancer patients with comet assay and micronucleus assay. *Mutat. Res*., **617**, 104–110.
29. Burgaz, S., Coskun, E., Demircigil, G. C., Kocabas, N. A., Cetindag, F., Sunter, O. and Edinsel, H. (2011) Micronucleus frequencies in lymphocytes and buccal epithelial cells from patients having head and neck cancer and their first-degree relatives. *Mutagenesis*, **26**, 351–356.
30. Chang, P., Li, Y. and Li, D. (2011) Micronuclei levels in peripheral blood lymphocytes as a potential biomarker for pancreatic cancer risk. *Carcinogenesis*, **32**, 210–215.
31. García-Quispes, W. A., Pastor, S., Galofré, P., Biarnés, F., Castell, J., Velázquez, A. and Marcos, R. (2013) Influence of DNA-repair gene variants on the micronucleus frequency in thyroid cancer patients. *Mutat. Res*., **750**, 34–39.
32. Baltaci, V., Kayikçioğlu, F., Alpas, I., Zeyneloğlu, H. and Haberal, A. (2002) Sister chromatid exchange rate and alkaline comet assay scores in patients with ovarian cancer. *Gynecol. Oncol*., **84**, 62–66.
33. Fan, R., Kumaravel, T. S., Jalali, F., Marrano, P., Squire, J. A. and Bristow, R. G. (2004) Defective DNA strand break repair after DNA damage in prostate cancer cells: implications for genetic instability and prostate cancer progression. *Cancer Res*., **64**, 8526–8533.
34. Jyoti, S., Khan, S., Naz, F., Ali, F. and Siddique, Y. H. (2013) Assessment of DNA damage by panmasala, gutkha chewing and smoking in buccal epithelial cells using alkaline single cell gel electrophoresis (SCGE). *Egypt. J. Med. Hum. Genet.*, **14**, 391–394.
35. Szeto, Y. T., Benzie, I. F., Collins, A. R., Choi, S. W., Cheng, C. Y., Yow, C. M. and Tse, M. M. (2005) A buccal cell model comet assay: development and evaluation for human biomonitoring and nutritional studies. *Mutat. Res*., **578**, 371–381.
36. Rojas, E., Valverde, M., Sordo, M. and Ostrosky-Wegman, P. (1996) DNA damage in exfoliated buccal cells of smokers assessed by the single cell gel electrophoresis assay. *Mutat. Res*., **370**, 115–120.
37. Valverde, M., del Carmen López, M., López, I., Sánchez, I., Fortoul, T. I., Ostrosky-Wegman, P. and Rojas, E. (1997) DNA damage in leukocytes and buccal and nasal epithelial cells of individuals exposed to air pollution in Mexico City. *Environ. Mol. Mutagen*., **30**, 147–152.
38. Speit, G. and Schmid, O. (2006) Local genotoxic effects of formaldehyde in humans measured by the micronucleus test with exfoliated epithelial cells. *Mutat. Res*., **613**, 1–9.
39. Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S. and Fenech, M. (2008) The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. *Mutat. Res*., **659**, 93–108.
40. Tolbert, P. E., Shy, C. M. and Allen, J. W. (1992) Micronuclei and other nuclear anomalies in buccal smears: methods development. *Mutat. Res*., **271**, 69–77.
41. Grover, S., Mujib, A., Jahagirdar, A., Telagi, N. and Kulkarni, P. (2012) A comparative study for selectivity of micronuclei in oral exfoliated epithelial cells. *J. Cytol*., **29**, 230–235.
42. Jyoti, S., Khan, S., Afzal, M., Naz, F. and Siddique, Y.H. (2013) Evaluation of micronucleus frequency by acridine orange fluorescent staining in buccal epithelial cells of oral submucosus fibrosis (OSMF) patients. *Egypt. J. Med. Hum. Genet*., **14**, 189–193.
43. Bloching, M., Hofmann, A., Lautenschläger, C., Berghaus, A. and Grummt, T. (2000) Exfoliative cytology of normal buccal mucosa to predict the relative risk of cancer in the upper aerodigestive tract using the MN-assay. *Oral Oncol*., **36**, 550–555.
44. Halder, A., Chakraborty, T., Mandal, K., Gure, P. K., Das, S., Raychowdhury, R., Ghosh, A. K. and De, M. (2004) Comparative study of exfoliated oral mucosal cell micronuclei frequency in normal, precancerous and malignant epithelium. *Int. J. Hum. Genet*., **4**, 257.
45. Fareed, M., Afzal, M. and Siddique, Y. H. (2011) Micronucleus investigation in buccal mucosal cells among pan masala/gutkha chewers and its relevance for oral cancer. *Biol. Med.*, **3**, 8–15.
46. Sellappa, S., Balakrishnan, M., Raman, S. and Palanisamy, S. (2009) Induction of micronuclei in buccal mucosa on chewing a mixture of betel leaf, areca nut and tobacco. *J. Oral Sci*., **51**, 289–292.
47. Anila, K., Kaveri, H. and Naikmasur, V. G. (2011) Comparative study of oral micronucleated cell frequency in oral submucous fibrosis patients and healthy individuals. *J. Clin. Exp. Dent*., **3**, e201–e206.
48. Palve, D. and Tupkari, J. V. (2008) Clinico-pathological correlation of micronuclei in oral squamous cell carcinoma by exfoliative cytology. *J. Oral. Maxillofac. Pathol.*, **12**, 2–7.
49. Khanna, S., Purwar, A., Singh, N. N., Sreedhar, G., Singh, S. and Bhalla, S. (2014) Cytogenetic biomonitoring of premalignant and malignant oral lesions by micronuclei assessment: a screening evaluation. *Eur. J. Gen. Dent*., **3**, 46.
50. Kamath, V. V., Anigol, P. and Setlur, K. (2014) Micronuclei as prognostic indicators in oral cytological smears: a comparison between smokers and non-smokers. *Clin. Cancer Invest. J*., **3**, 49.
51. Silverman, S., Bhargava, K., Smith, L. W. and Malaowalla, A. M. (1976) Malignant transformation and natural history of oral leukoplakia in 57,518 industrial workers of Gujarat, India. *Cancer*, **38**, 1790–1795.
52. Mehta, F. S., Shroff, B. C., Gupta, P. C. and Daftary, D. K. (1972) Oral leukoplakia in relation to tobacco habits. A ten-year follow-up study of Bombay policemen. *Oral Surg. Oral Med. Oral Pathol*., **34**, 426–433.
53. Bokor-Bratić, M. and Vučković, N. (2002) Cigarette smoking as a risk factor associated with oral leukoplakia. *Arch. Oncol*., **10**, 67–70.
54. Wu, P. A., Loh, C. H., Hsieh, L. L., Liu, T. Y., Chen, C. J. and Liou, S. H. (2004) Clastogenic effect for cigarette smoking but not areca quid chewing as measured by micronuclei in exfoliated buccal mucosal cells. *Mutat. Res*., **562**, 27–38.
55. Konopacka, M. (2003) Effect of smoking and aging on micronucleus frequencies in human exfoliated buccal cells. *Neoplasma*, **50**, 380–382.
56. Mahimkar, M. B., Samant, T. A., Kannan, S. and Patil, T. (2010) Influence of genetic polymorphisms on frequency of micronucleated buccal epithelial cells in leukoplakia patients. *Oral Oncol*., **46**, 761–766.
57. Tilakaratne, W. M., Klinikowski, M. F., Saku, T., Peters, T. J. and Warnakulasuriya, S. (2006) Oral submucous fibrosis: review on aetiology and pathogenesis. *Oral Oncol*., **42**, 561–568.
58. Chakradeo, P. P., Nair, J. and Bhide, S. V. (1994) Endogenous formation of N-nitrosoproline and other N-nitrosamino acids in tobacco users. *Cancer Lett*., **86**, 187–194.
59. Murdia, U. S., Mehta, F. J. and Bhide, S. V. (1982) Nitrate reductase activity and nitrite levels in the saliva of habitual users of various tobacco products. *Food Chem. Toxicol*., **20**, 269–271.
60. Joshi, M. S., Verma, Y., Gautam, A. K., Parmar, G., Lakkad, B. C. and Kumar, S. (2011) Cytogenetic alterations in buccal mucosa cells of chewers of areca nut and tobacco. *Arch. Oral Biol*., **56**, 63–67.
61. Rajendran, R. (2005) Oral lichen planus. *J. Oral. Maxillofac. Pathol*., **9**, 3.
62. Mukherjee, M., Gyawali, P., Thakur, R. K., Shenoy, R. P. and Rao, A. (2011) Assessment of frequency of micronucleated exfoliated buccal cells in relation to oxidative stress in oral lichen planus in coastal Karnataka, India. *Asian Pac. J. Trop. Dis.*, **1**, 287–288.
63. Zainol, M., Stoute, J., Almeida, G. M., Rapp, A., Bowman, K. J. and Jones, G. D. (2009) Introducing a true internal standard for the Comet assay to minimize intra- and inter-experiment variability in measures of DNA damage and repair. *Nucleic Acids Res*., **37**, e150.
64. Titenko-Holland, N., Moore, L. E. and Smith, M. T. (1994) Measurement and characterization of micronuclei in exfoliated human cells by fluorescence in situ hybridization with a centromeric probe. *Mutat. Res*., **312**, 39–50.