Evaluation of DNA damage in oral precancerous and squamous cell carcinoma patients by single cell gel electrophoresis

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ABSTRACT

Context: Single cell gel electrophoresis (SCGE) or “comet assay” is a rapid and very sensitive fluorescent microscopic method for detecting various forms of DNA damage at individual cell level.

Aims: The aim of the present study was to detect the extent of DNA damage in oral cancer, oral submucous fibrosis (OSMF) and leukoplakia in comparison to normal individual.

Settings and Design: A total of 44 consecutive patients with oral cancer (n=26), leukoplakia (n=12) and OSMF (n=6) and 10 healthy normal volunteers with normal oral epithelia (controls) were recruited from Dr. R. Ahmed Dental College and Hospital and were assessed for the extent of DNA damage using SCGE following clinical diagnosis.

Materials and Methods: Peripheral blood was collected by venepuncture and comet assay was performed using SCGE. Mean tail length was compared between diagnostic groups and between different oral habit groups using t-tests and analysis of variance (ANOVA). Pearson’s product moment correlation was used to examine the linear association between the extent of DNA damage and oral habit pack-years. Scheffe’s pair-wise test was employed to adjust for multiple comparisons.

Results: None of the controls were associated with any oral habits. Mean (±SD) tail lengths (in µm) for cancer (24.95 ± 5.09) and leukoplakia (12.96 ± 2.68) were significantly greater than in controls (8.54 ± 2.55, P<0.05). After adjustment, well-, moderately, and poorly differentiated carcinomas had significantly greater tail length than controls. Whereas the extent of DNA damage in cancer cases was significantly greater in leukoplakia than in compared to OSMF (11.03 ± 5.92), the DNA damage in latter was not different from controls. DNA damage for people with any oral habit (19.78 ± 7.77) was significantly greater than those with no habits (8.54 ± 2.55; P<0.0001).

Conclusions: DNA damage measured by SCGE is greater in leukoplakia and squamous cell carcinoma, but not in OSMF. Deleterious oral habits are also associated with greater DNA damage.

Key words: Comet assay, damage, DNA, leukoplakia, single cell gel electrophoresis, squamous cell carcinoma, submucous fibrosis

Oral squamous cell carcinoma has a multifactor etiology. Factors such as genetic, environmental and gene–environment interactions, viral, and behavioral (smoking, alcohol) have been implicated in the etio-pathologic continuum of oral cancer including recent population-based studies. One of the important hallmarks for cancer progression is DNA damage, resulting either from various carcinogens accumulating from etiologic influences or due to genetic errors. If detectable and quantifiable, these may contribute toward an easy detection and prediction system for oral cancer development and prognosis. Molecular and genetic techniques have enabled us to unravel some of the critical events associated with development of oral cancer and precancer. Examining associations of DNA damage with environmental factors and factors related to personal habits is an important challenge for molecular epidemiology, especially to develop better early detection tests and provide better prediction tests for oral cancer occurrence and progression.

The “comet assay”, also called single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells, originally developed by Östling and Johansson in 1984. Singh et al., later modified this technique in 1988, when it became...
The name of the assay comes from the image of the electrophoresis gel which resembles a "comet" with a distinct head and tail. The cell with DNA damage appears in the form of "comet" while undamaged cell appears as a halo. The head is composed of intact DNA, while the tail consists of damaged (single-strand or double-strand breaks) or broken pieces of DNA.

The SCGE has been used for evaluating DNA lesions and is used to detect DNA damage caused by double-strand breaks, single-strand breaks (SSB), alkali labile sites, oxidative base damage, and DNA cross-linking with DNA or protein. The SCGE is also used to monitor DNA repair by living cells and is applicable to any eukaryotic organism and cell type. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. The comet assay is inexpensive once the laboratory infrastructure has been set in place, and gives results within a few hours and it is an appropriate tool for environmental monitoring. Though handling of samples, for example, proper protection from light and less shearing of blood samples are some essential criteria that should be taken care of.

Although cellular changes in peripheral blood in a diverse range of malignancies have been reported in megakaryocytes, monocytes and in polymorphonuclear leukocytes, the DNA SSB have not been studied in detail. Similarly, although there is evidence that deleterious oral habits such as smoking, alcohol consumption and betel quid chewing are associated with oral cancer, no study has examined the association of DNA damage with deleterious oral habits such as smoking, pan chewing, and chewing tobacco.

**MATERIALS AND METHODS**

Study subjects were recruited from Dr. R. Ahmed Dental College and Hospital in Kolkata, India, following standard clinical diagnostic criteria and under their informed consent. The study was approved by an ethics review committee of the institute. A total of 44 consecutive patients with oral cancer (n=26), leukoplakia (n=12) and OSMF (n=6) and 10 healthy normal volunteers with normal oral epithelia (controls) were assessed for the extent of DNA damage using SCGE. Complete medical history was taken and oral habits were recorded in detail using a detailed questionnaire administered to all subjects. Detailed oral examination was carried out by a trained clinical oral pathologist. The lesion diagnoses were confirmed histopathologically. Whole blood was collected from antecubital vein, labeled and stored in tubes [with ethylenediaminetetraacetic acid (EDTA)], which were kept in a flask with ice and protected from light until processed in the laboratory.

**SCGE of DNA**

Phosphate buffered saline (PBS) suspended peripheral blood cells were embedded in a thin agarose gel (1% low melting) on a microscope slide. All cellular proteins were then removed from the cells by lysing with a lysis buffer [2.5 M NaCl, 0.1 M Na2 EDTA and 10 mM Trisma base, 1% TritonX and 10% dimethyl sulfoxide (DMSO)] and refrigerated overnight. DNA was allowed to unwind under alkaline conditions. Electrophoresis was performed for 20 minutes under alkaline condition (300 mM NaOH, and 1 mM EDTA, pH>13) at 280 mA and 24 V (−0.74 V/cm). The broken DNA fragments or damaged DNA undergoing electrophoresis migrate away from the nucleus. In this process, the smallest fragments travel the farthest. Following electrophoresis, the slides were neutralized using neutralizing buffer (0.4 M Tris, pH 7.5). Thereafter, following staining with a fluorescent dye (ethidium bromide 2 µg/ml), the DNA “comet” was easily visualized using a fluorescent microscope (Leica DM 3000) and the head and tail lengths were measured in 50 randomly selected cells per slide using Leica QWinPlus digital image processing and analysis software, Leica Microsystems, Switzerland. The DNA damage was evaluated by the length of the comet’s tail for each cell.

**Statistical analysis**

This cross-sectional study was analyzed as a traditional case-control study with the extent of DNA damage as the main outcome. Oral habits were dichotomized as present/absent and a second variable of oral habit pack-years of exposure was also examined. Mean tail length was compared between diagnostic groups and between different oral habit groups using t-tests and analysis of variance (ANOVA). Pearson’s product moment correlation was used to examine the linear association between the extent of DNA damage and oral habit pack-years. Scheffe’s pair-wise test was employed to adjust for multiple comparisons. All statistical analyses were done using SAS® V8.2 statistical software package (PC-SAS) (Cary, NC, USA).

**RESULTS**

Mean (±SD in years) age of controls (36 ± 16.4) was greater than OSMF (30.0 ± 11.2) although lesser than leukoplakia (44.0 ± 8.6) and oral cancer (54.3 ± 8.5) patients. Overall, 85% of the subjects in the study sample were men; 82% had at least one deleterious oral habit (43% smoked bidi, 17% smoked cigarette, 15% chewed pan, and 15% chewed tobacco); 48% had oral cancer; 22% leukoplakia, 11% OSMF and 19% were controls with no oral lesions and having normal oral epithelium. Overall, 46.4% had office-based jobs; 37% were farmers or laborers and the rest (17%) either stayed at home or were students attending university.

Figures 1–4 show the representative “comets” in normal epithelium, leukoplakia, OSMF and oral cancer. Table 1
provides a composite description of DNA damage and extent of oral habits (in pack-years) in different lesion diagnostic groups. The mean (±SD) tail lengths (in µm) for oral cancer (24.95 ± 5.09) and leukoplakia (12.96 ± 2.68) were statistically significantly more than that of controls (8.54 ± 2.55, *P*<0.05) in bivariate analysis. Mean tail length in OSMF, although higher than controls (11.03 ± 5.92), was not significantly different. Extent of bidi smoking and pan chewing (measured in pack-years) were substantially greater in oral cancer subjects compared to others. Substantial pack-years of bidi and cigarette smoking exposure were evident among leukoplakia subjects. None of the OSMF subjects in this study reported chewing pan although they reported substantial exposure to chewing tobacco.

Table 2 describes the extent of DNA damage among subjects with different deleterious oral habits. Extent of DNA damage for people with any oral habit (19.78 ± 7.77) was significantly greater than those with no habits at all (8.54 ± 2.55; *P*<0.0001). Similarly, those smoking bidi, cigarette, or pan had significantly greater DNA damage compared to subjects not having those habits in the respective habit groups. Pearson’s correlation between tail length and oral habits suggests moderate positive linear correlation between bidi

Table 1: Mean (standard deviation) comet length (DNA damage – tail length) and oral habits in pack-years in oral cancer and precancer groups

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Tail length (µm)</th>
<th>Bidi (pack-years)</th>
<th>Cigarette (pack-years)</th>
<th>Pan (pack-years)</th>
<th>Tobacco (pack-years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Normal</td>
<td>10</td>
<td>8.54</td>
<td>2.55</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OSMF</td>
<td>6</td>
<td>11.03</td>
<td>5.92</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>LK</td>
<td>12</td>
<td>12.96*</td>
<td>2.69</td>
<td>89.2</td>
<td>139.1</td>
</tr>
<tr>
<td>Cancer</td>
<td>26</td>
<td>24.95*</td>
<td>5.02</td>
<td>369.6</td>
<td>272.7</td>
</tr>
</tbody>
</table>

*Statistically significantly different from normal (*t*-test) at 0.0001 level in bivariate analysis.
Table 2: Tail length (µm) by oral habits group

<table>
<thead>
<tr>
<th>Habit type</th>
<th>Presence of oral habit</th>
<th>Correlation††</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>Bidi</td>
<td>23</td>
<td>22.32†</td>
</tr>
<tr>
<td>Cigarette</td>
<td>9</td>
<td>14.80*</td>
</tr>
<tr>
<td>Pan</td>
<td>8</td>
<td>25.46*</td>
</tr>
<tr>
<td>Tobacco</td>
<td>8</td>
<td>13.86</td>
</tr>
</tbody>
</table>

*Statistically significantly different from those without the corresponding habit (t-test) at 0.001 level in bivariate analysis. **P=0.06 (bivariate analysis, t-test). †Pearson’s correlation between tail length (µm) and presence of oral habit. ‡Moderate positive linear correlation.

smoking exposure in pack-years and tail length (r=0.52), and between pan chewing (in pack-years) and tail length (r=0.41).

Table 3 shows the ANOVA source table for modeling tail length differences between controls, leukoplakia, OSMF and leukoplakia after adjusting for deleterious oral habits such as bidi smoking, tobacco chewing, pan chewing and cigarette smoking. The overall model was better than an intercept-only model (P<0.0001). This ANOVA model had good explanatory power explaining 76% of the variability in the data (R²=0.757). After adjustment for oral habits, oral cancer subjects had significantly greater tail length than that of controls. Extent of DNA damage of cancer cases was also significantly greater than in leukoplakia. However, tail length in OSMF (11.03 ± 5.92) or leukoplakia (12.96 ± 2.69) patients was not significantly different from that of controls. Upon removing OSMF from the diagnostic lesion groups and the non-contributing exposure factors (cigarette smoking and tobacco chewing), the difference in tail length between leukoplakia and controls came close to the statistical significance level.

DISCUSSION

Several studies have demonstrated an independent association of deleterious oral habits such as smoking, betel quid chewing and chewing tobacco with occurrence of oral cancer.[12] The precancerous conditions such as leukoplakia, characterized by a white patch, mainly associated with tobacco smokers, and OSMF, characterized by deposition of collagen and marked blanching of oral cavity, leading to inability to open mouth, mainly associated with smokeless tobacco and betel quid chewers, have a greater prevalence in our country. Both of these conditions have high cancer turnover potentiality and if detected early can be prevented and treated successfully. With present advances in genotoxicologic studies, extent of DNA damage provides a platform to determine the cancer progression.[13] The association of these oral habits with DNA damage has never been clearly established. Moreover, comet assay provides a generalized picture of a population that shows whether it is under environmental carcinogenic stress, and may become an epidemiologic asset. We have been able to demonstrate in this study that DNA damage is associated with oral cancer and leukoplakia, and also that DNA damage is associated with deleterious oral habits. Bidi smoking and pan chewing or presence of any deleterious oral habit led to DNA with greater tail length.

Exploring these relationships a little further, we found moderate linear relationship between smoking and pan chewing, and comet tail length, providing general evidence that greater the exposure to these habits, greater would be the extent of DNA damage and vice versa. However, the difference in comet tail length between those chewing and not chewing tobacco did not achieve statistical significance in this study just as the Pearson’s coefficient showed a negative, but strong correlation. But comparing the extent of DNA damage of tobacco chewers with those with no oral habits presented a somewhat different scenario. We attribute these discrepancies to the composition of comparison groups because those reporting not chewing tobacco had several other habits that confounded the relationship between tobacco chewing and extent of DNA damage. We did not have enough mutually exclusive oral habit groups to allow a meaningful comparison of independent effect of each oral habit. Similar explanation also holds for the association of cigarette smoking with the extent of DNA damage.

In multivariable analyses, ANOVA models suggested that comet tail length was significantly greater in oral cancer compared to other groups even after adjusting for all oral habits that were included in the models. This model was better than an intercept-only model and also explained about 76% of the variation. Although we demonstrated significantly greater tail length in leukoplakia compared to controls in bivariate analysis, this was not obvious in multivariable analyses after adjusting for multiple comparison. However, upon further modeling, we found that by removing oral habits that did not contribute much to the model (i.e. keeping only the main contributor – bidi smoking), the R² value increased to 78% and comet tail length difference between leukoplakia and controls came very close to becoming statistically significant. Although we could not specifically demonstrate that extent of DNA damage was greater in leukoplakia compared to controls after adjusting for oral habits, we attribute this to the low power in this study.

The present study has some limitations that include its small sample size, and because of this we observed a peculiar problem in data analysis as none of the controls reported any
This observation did not allow for certain comparisons to be carried out. However, we noticed that extent of oral habits measured as pack-years of exposure were substantially greater for oral cancer and leukoplakia patients compared to those not exposed to the corresponding oral habit. Bivariate analyses demonstrated that extent of DNA damage in oral cancer and leukoplakia were greater than in controls and pack-year of exposure were generally greater in oral cancer. Despite the limitations, we have been able to demonstrate that DNA damage in blood cells measured by SCGE is greater in oral cancer and leukoplakia, but not in OSMF. Deleterious oral habits are also associated with greater DNA damage.

Although none of the evidence in this cross-sectional and small study per se provides an opportunity to consider temporal associations and infer causality, there is preponderance of evidence in published literature to discuss potentially causal associations in the context of this study. One of the key factors to consider is that the DNA damage demonstrated in our study is in T-cells in the blood which belongs to a distinctly different biological compartment than the site of the lesion. From a potentially etiological mechanism explanation standpoint, the biological effect(s) of oral habits on oral tissues would take place through direct interaction between the exposure agent and/or a subsequent exposure through systemic absorption and release through subepithelial capillaries in the submucosa or through secretion into either saliva or gingival crevicular fluid. Therefore, it is intuitive to consider that substantial DNA damage demonstrated in the blood compartment is an indirect evidence of potentially greater damage in local tissue (oral epithelium). Measuring the extent of DNA damage directly in oral epithelium would provide direct evidence of association of DNA damage in oral epithelium with these deleterious oral habits to develop an etiological explanation. However, measuring the extent of DNA damage in blood cells could provide for the possibility of developing an easy detection test because blood is collected in all routine investigations and no extra invasion would need to be performed for such a test, should it become available in the future.

Studies have demonstrated direct lesion tissue genetic damage in leukoplakia with increased frequency of loss of heterozygosity. Therefore, demonstration of DNA damage using SCGE in oral epithelium will expand the possibility of developing early detection tests using exfoliative cytology techniques. Suitability of comet assay performed on white blood cells as a rapid test in biomonitoring occupational exposure to DNA-damaging agents across several industries has been extensively reviewed by Møller et al. The authors concluded that in general, the comet assay is considered a suitable and fast test for DNA-damaging potential in biomonitoring studies. Genetic changes in margins of resections of cancer/precancer may also be an important determinant of success of intervention in these cases. These are some of the potential situations where demonstrating DNA damage using SCGE may lead to further advancement in our understanding of the biology of oral cancer and precancer for developing possible worthy early detection tests.

**REFERENCES**


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