Identification of genetic instability in peripheral blood lymphocyte of oral squamous cell carcinoma patients assess by comet assay

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Abstract

Context: Studies established that human cancer is principally a genetic disease; it arises as accumulation of a set of genetic changes. In the pathogenesis of cancer, genetic instability is the sequential event to a carcinogenic stimulus resulting in various genomic changes including DNA damage.

Aims: To assess genetic instability, as susceptibility to DNA damage, we used single-cell gel electrophoresis (comet assay) to study double strand breaks in associated with the risk of oral squamous cell carcinoma (OSCC).

Materials and Methods: We used comet assay to measure double strand break in individual peripheral blood lymphocytes from 50 individuals with OSCC and 30 healthy control subjects. All personal information was gathered from subjects including tobacco history. DNA damage was visualized as comet assay and quantified by movement of damaged strands as length of tail.

Results: Study results of OSCC patients were observed in relation to clinical staging and histological grading of carcinoma. On the basis of clinical observation, cases were grouped in to Stage I, Stage II, Stage III and Stage IV. No stage I cases were in study sample. The mean DNA damage migration length was observed 4.600 ± 0.4613 μm in stage II, whereas in Stage III and Stage IV, it was observed to be 4.961 ± 0.5620 μm and 4.883 ± 0.410 μm, respectively. The DNA damage length in histological grades of squamous cell carcinoma patients in Grade I was 4.6437 ± 0.3061 μm and Grade II was 5.3533 ± 0.3831 μm. In comparison with control group and squamous cell carcinoma group, it was observed in the range of 0.02–0.36 μm and varied from 4.04 to 5.84 μm range, respectively. Thus, the results were statistically significant with the histological grading of OSCC. Statistical Analysis: Unpaired t test and ‘ANOVA’ test are used for statistics.

Conclusion: The amount of DNA strand breaks in peripheral lymphocytes are measured by comet assay which is associated with relative risk of OSCC.

Keywords: Comet assay, DNA damage, DNA strands, gel electrophoresis, lymphocyte, squamous cell carcinoma

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INTRODUCTION

Oral cancer globally is a serious growing problem and occupies the sixth most common cancer worldwide.\[1-3\] New cases of oral cancer exceed 410,000 annually and account for between 1% and 5% of all cancer worldwide, but in India may be as high as 15%–40% of all cancer registered.\[4,5\] Oral squamous cell carcinoma (OSCC) accounts for 3%–5% of all malignancies. The World Health Organization predicts a continuing worldwide increase in the incidence of oral cancer.\[6\]

A number of factors contribute to the cause of oral cancer. In countries like India where the standard of living is low, oral cancer has the highest rate and is the most common type of cancer in males. Various irritants such as tobacco, (chewed, inhaled, or swallowed) and alcohol are clearly important.

In carcinogenesis, the initial step involves more or less alteration in the DNA of the cell.\[7\] Malignancy-associated changes were also noted in blood leukocytes, megakaryocytes and monocytes.\[8-10\]

A variety of methods have been developed for detecting DNA strand damage such as micronuclei, sister-chromatid exchange assay and snapshot of DNA strand break single-cell gel electrophoresis.

Single-cell gel electrophoresis or comet assay is a more recent method to detect DNA single strand breaks (SSB) and alkali-labile sites by measuring the migration of DNA from immobilized nuclear DNA and have an advantage over other methods like it can be done on any eukaryotic cell, economical, simple and fast.\[11,12\]

The aim of this study was to find out the DNA damage index in peripheral blood lymphocytes in patients with OSCC and to compare the DNA damage index between patients of OSCC and the healthy control group.

MATERIALS AND METHODS

The study consisted of 50 diagnosed patients of OSCC and 30 healthy individuals without any tobacco-related habit with a detailed case history. All participants enrolled with prior consent and detailed case history filters the prerequisite for study.

Under all aseptic conditions, 2 ml of venous blood was drawn from OSCC patients and healthy subjects with the help of a disposable needle and syringe. It was then transferred into sterile glass bottles containing 3.8% sodium citrate, used as an anticoagulant.

The clinical staging of patients suffering from OSCC was done based on site, size and extent of the lesion and involvement of lymph nodes as per the TNM classification given by the American Joint Committee for cancer staging and end result reporting (AJCCS) in 2018. Histopathological grading of squamous cell carcinoma was done as Grade I (well differentiated), Grade II (moderately differentiated) and Grade III (poorly differentiated) according to the malignancy grading system.\[13\]

Preparation of slides for single-cell gel electrophoresis

Lymphocytes were isolated from the sample of blood by standard centrifugation at 400 × g at room temperature for 30 min with lymphocyte separating medium\[14\] (HiSep™ LSM 1077).

Lymphocyte cell suspension was added to 140 μl of 0.5% low melting agarose (26°C–30°C).

Plane microscopic slides were covered with 220 μl of 0.5% normal melting agarose (melting point 36°C ± 1.5°C) in Ca²⁺- and Mg²⁺-free phosphate-buffered saline and immediately covered with a coverslip and placed in the freezer at 4°C for 3 min to solidify. After gently removing the coverslip, 140 μl of 0.5% melting agarose in which blood sample is loaded at 37°C was rapidly pipetted on the first layer of agarose and kept at 4°C for 4 min to solidify. The second layer was overlaid. Similarly, third layer of low melting agarose (140 μl of 0.5%) placed on slide and kept in freezer at 4°C to solidify. Then, the slides were immersed in cold (5°C–8°C) lysing solution (2.5 M Nacl, 100 mM disodium EDTA, 10 mM Tris at pH 10) to which the freshly prepared 1% Triton × 100 and 10% dimethyl sulfoxide were added and the slides were left over night at 4°C.

The slides were then removed from the lysing solution and left in alkaline buffer for 20 min prior to electrophoresis in order to allow unwinding of DNA.

Then, the slides were placed in a horizontal gel electrophoresis system. The tank was filled with fresh electrophoresis buffer (1 mM disodium EDTA and 300 mM NaOH).

After electrophoresis, the slides were washed by flooding with neutralizing buffer (0.4 M Tris, pH 7.5) and left for 10 min so as to neutralize the charged ions. This procedure was repeated twice. After neutralization, slides were stained with silver nitrate.

Cells with DNA damage appeared as comets [Figure 1]. Total image length (nuclear and migrating DNA) and
the image (nuclear) diameter were measured using an oculometer fitted to the eyepiece after standardization with a stage micrometer. The DNA tail lengths were measured by subtracting the diameter from the total length.

RESULTS AND OBSERVATIONS

The present study on the basis of clinical staging grouped as 3 cases (6%) of Stage II, 20 cases (40%) of Stage III and 27 cases (54%) of Stage IV. Not a single case was observed in Stage I. Histological grading was done of all 50 patients as 32 cases (64%) of Grade I and 18 cases (36%) of Grade II squamous cell carcinoma. There was no case of Grade III carcinoma.

The DNA damage length was observed in the control group [Figure 2] which was in the range of 0.02–0.36 μm, whereas it varied from 4.04 to 5.84 μm range in squamous cell carcinoma group. The mean DNA damage length observed in the control group was found to be 0.0987 ± 0.0546 μm, whereas in the squamous cell carcinoma group, it was observed to be 4.8992 ± 0.4781 μm, which were statistically significant [Table 1].

In squamous cell carcinoma group, DNA-SSB in different clinical stages was observed as follows. There were no cases in Stage I. In Stage II, the mean DNA damage length was observed 4.600 ± 0.4613 μm, whereas in Stage III and Stage IV, it was observed to be 4.961 ± 0.5620 μm and 4.883 ± 0.410 μm, respectively [Table 1].

The DNA damage length in histological grades of squamous cell carcinoma patients was as follows: The mean DNA damage length in Grade I was 4.6437 ± 0.3061 μm, whereas in Grade II, it was 5.3533 ± 0.3831 μm. There was no case in Grade III squamous cell carcinoma patients [Table 1].

The difference between mean DNA damage length values of different histological grades was calculated and statistical evaluation was done using an unpaired t-test. The DNA damage length was found to increase from Grade I and Grade II, and the difference was extremely statistically significant [P < 0.0001; Table 1].

The values of DNA damage length were also compared within different clinical stages of squamous cell carcinoma, the mean difference was calculated and the statistical analysis was done using one-way “ANOVA” test, the values were observed, but the difference was not statistically significant [P = 0.47; Table 1] since there was no case in Stage I.

DISCUSSION

Oral cavity cancer is one of the most lethal cancers, ranking as the sixth-leading cause of cancer mortality.
Majority of new cases of intraoral cancer are discovered only when patients become symptomatic. More than 80% of these cancers are in advanced stages.

Alterations in peripheral blood cells in a diverse range of malignancies are well known. The occurrence of DNA damage in these cells is a recent finding. The increased frequency of DNA-SSB was observed in peripheral blood leukocytes in the patients with precancerous and cancerous lesions of the uterine cervix.[9,17]

Lymphocytes play an important role in defense mechanism. Hence, the above observations lead us to study the DNA-SSB in lymphocytes of OSCC patients. A solitary study has been done in OSCC by Rao et al.[17] where DNA damage in peripheral blood leukocytes was examined.

The single-cell gel electrophoresis assay utilizes nucleated cells, which are sandwiched between different agarose layers and subsequently subjected to lytic treatment at high salt concentrations. After electrophoresis under alkaline conditions and staining with fluorescent dyes or silver nitrate, nuclei with damage can be quantified under a microscope using an ocular micrometer.

DNA-specific dyes used for comet visualization depend on largely investigator-specific needs and presumably have little effect on assay sensitivity or reliability.[11] The fluorescent dyes used most frequently are ethidium bromide,[18] propidium iodide, 4, 6-diamidino-2 and phenylindole (Lee and Steinert, 2003)[11] and SYBR Green (Singh et al. 1998).[18] The common nonfluorescent staining technique for visualization of comet assay is by silver nitrate staining technique.[19,20] In the present study we used silver nitrate stain due to its simple staining technique and ready availability. The stained slides were observed in monocular microscope in 100X to identify DNA damage.

In the present study, the squamous cell carcinoma cases were distributed in the 3rd, 4th, 5th, 6th and 7th decades. The maximum number of cases were observed to involve alveolar ridge (20 cases) followed by buccal mucosa (15 cases), which was the actual placement of tobacco quid in most of the patients.

In the present study, DNA damage index in peripheral blood lymphocyte of the control group was observed in the range of 0.02–0.36 μm with a mean damage index of 0.0987 ± 0.0546 μm. The study done by Rao et al.[17] showed quite similar results in healthy control groups as 0.4900 ± 0.1323.

In the present study, the DNA damage index was evaluated according to histological grades of squamous cell carcinoma. The mean levels observed were 4.643 and 5.353 μm for Grade I and Grade II, respectively. These levels were similar to those obtained by Rao et al.[17] which were 4.352 and 5.131 μm for Grade I and Grade II squamous cell carcinoma, respectively. The overall level of DNA damage index was observed to be in the range of 4.04 to 5.84 μm, which was in the range of those obtained by Rao et al.[17] which were 3.72 to 6.84 μm.

Another observation in the study of Rao et al.[17] was that the DNA damage length increased significantly from Grade I, Grade II and Grade III respectively in squamous cell carcinoma group. Thus, the DNA damage detected by single-cell gel electrophoresis or comet assay correlated well with the histological grading of squamous cell carcinoma. In the present study, similar results were obtained.

The patients were also grouped according to clinical stages. The DNA damage length was observed to be more in Stage III (40961 ± 0.562 μm) as compared to Stage IV (4.883 ± 0.410 μm) and Stage II (4.60 ± 4.61 μm). The results were not in increasing order as in clinical stages. Hence, the difference was not statistically significant. There were no cases in Stage I, and hence, a comparison could be done between I and II and III and IV stages. The results obtained by Venkateswara R et al.[17] were highly significant between Stage I and Stage II, Stage I and Stage IV, Stage II and Stage IV and Stage III and Stage IV, while between Stage I and III and II and III, it was not very significant.

CONCLUSION

Genetic instability in the form of DNA damage appears to be associated with an increased relative risk of oral cancer. Measuring single strand break with comet assay is a reliable approach for determining susceptibility and genetic instability in an individual toward cancer. Patients suffering from OSCC show a remarkable difference in the degree of single strand breaks as a result of genetic instability. Control subjects show a constant degree of SSB in comparison with OSCC patients.

Single-cell gel electrophoresis is a simple method to detect single strand breaks in the DNA of individual cells through peripheral blood lymphocyte. OSCC is known to have DNA damage in oral epithelial cells and in peripheral blood leukocytes. Although this study may not enough to demonstrate the progression and prognosis of disease, large population-based studies are required to validate comet assay utility in clinical practice.
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Conflicts of interest
There are no conflicts of interest.

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