Genetic Changes in Oral Premalignant Lesion, Condition, and Oral Squamous Cell Carcinoma - A Study Based on Inhibition of G2M Phase by Colchicines

Abstract
Aims: To assess the percentage of cell arrested in G2M phase by colchicine in the early detection and prognosis of oral cancer, by comparison of oral squamous cell with leukoplakia and oral submucous fibrosis (OSMF) using flow cytometry analysis. Materials and Methods: Biopsy samples 5 each of clinically diagnosed and histopathologically confirmed case of leukoplakia, OSMF, and squamous cell carcinoma (SCC) are included in the study. Results: Colchicines significantly inhibited the growth of oral SCC-9 cell line by arresting G2M phase of the cell cycle (94.90%). This cell cycle result indicated that doubling of DNA is a characteristic feature of cancer. Colchicines significantly inhibited the G2M phase of the cell cycle in leukoplakia (33.51%) as compared to control (23.60%). Colchicines marginally inhibited the G2M phase of the cell cycle in OSMF (31.83%) as compared to control (28.36%). Colchicines significantly inhibited the G2M phase of the cell cycle in SCC (63.05%) as compared to control (26.40%). Conclusion: DNA analysis by inhibition of G2M phase of cell cycle using colchicine facilitates early detection and also helps determine the prognosis of leukoplakia, OSMF, and oral cancer.

Keywords: Colchicines, flow cytometry, G2M phase, leukoplakia, oral submucous fibrosis, squamous cell carcinoma

Introduction
Oral squamous cell carcinoma (OSCC) is the sixth common cancer in the world, with high incidence and mortality rate in the Asian subcontinent. OSCC is the most common malignancy found in the oral cavity, generally in areas such as tongue, buccal mucosa, and gingiva. OSCC has a multifactorial etiology including genetics, environmental and gene–environment interactions, and viral and behavioral factors such as smoking and alcohol. \(^1\)

The important cause for cancer prognosis is DNA damage. A variety of treatment modalities, including surgical intervention, radiotherapy, chemotherapy, and gene therapy have been developed. However, the survival rate of patients with oral OSCC has not significantly improved. In this study, we have used colchicine to inhibit the growth of oral cancer cells of biopsy sample compared with line SCC-9 through cell cycle analysis by fluorescent-activated cell sorting (FACS). Cell cycle examination is a method in cell biology that employs flow cytometry to differentiate cell in different phases of the cell cycle. Cell cycle anomalies can result from various kinds of cell damage, for example, DNA damage. Further possible reason for anomalies includes lack of nutrients, for example, after serum deprivation.\(^2\)

Before examination, the cells are permeabilized and treated with a fluorescent dye usually propidium iodide (PI) stain DNA quantitatively; the fluorescence intensity of the stained cell at certain wavelengths will therefore correlate with the amount of DNA they contain. As the DNA content of cell duplicates during the S phase of the cell cycle, the relative amount of cell in the G\(_0\) phase and G\(_1\) phase (before S phase), in the S phase, and in the G\(_2\) phase and M phase (after S phase) can be determined, as the fluorescence of cell in the G\(_2\)/M phase will be twice as that of cell in the G\(_0\)/G\(_1\) phase.\(^3\)

Cells and fixed cells tend to stick together; these aggregated cells have

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to be excluded from analysis through a method called doublet discrimination. Hence, this is important because a doublet of the cell in the $G_0/G_1$ phase has the same total amount of DNA and thus the same fluorescence intensity as a single cell in the $G_2/M$ phase. $G_0/G_1$ doublets would therefore create false positives result for $G_2/M$ cells.\cite{4}

Materials and Methods

Aims and objectives

To assess the percentage of the cell arrested in G2M phase of cell cycle using colchicine, for the early detection and prognosis of oral cancer. Comparison of OSCC with leukoplakia and oral submucous fibrosis (OSMF) using flow cytometry analysis was carried out.

Biopsy sample for each clinically diagnosed and histopathologically confirmed case of leukoplakia, OSMF, and SCC are included in the study. Biopsy of tissue sample is minced with scissors into small pieces <1 mm in diameter and then either transferred to a stirred flask at 37°C with 0.2% collagenase to disaggregate into single cells.

Procedure

We cultured $3 \times 10^5$ cells (SCC9 and isolated cells from biopsy tissues) in a p-35 plate 2 ml of complete media. After keeping overnight or 24 h of incubation, the cell plate was washed one time with 1× phosphate-buffered saline (PBS). Cells are serum starved for 24 h. Serum containing media with drugs are (colchicine; 10 µM) incubated for 24 h. After treatment, we collected both floating and attached cells by trypsin-ethylenediaminetetraacetic acid (EDTA). We pelleted $3 \times 10^5$ cells/ml at 1500 rpm for 5 min at room temperature and discarded the supernatant. We resuspended the cell pellets gently with two washes in 1× PBS. Cell pellets were fixed overnight 4°C in a 500 µl of 70% ethanol (Option: Fixing solution contains 15% fetal bovine serum and 15% PBS in 70% of ethanol). After overnight fixing, we centrifuged the cell at 1500 rpm for 5 min at room temperature and discarded the supernatant. Cell pellets washed two times with cold 1× PBS cell were incubated for 30 to 60 min at room temperature in 500 µl of PI solution containing 0.05 mg/ml PI, 0.1 mm EDTA, and 0.05 mg/ml RNase in PBS. The percentage of cell in various stage of cell cycle in the plant extracted and treated and untreated populations was determined using FACS.

Reagents

PI: Cat# P4865, Sigma; Stock 1 mg/ml, working solution is 0.05 mg/ml. EDTA: Cat# P120-500, Fisher; Stock 5 mm, working solution is 1 mm. RNase A: Cat# 109169, Boehringer Mannheim GmbH; Stock 5 mg/ml, working solution is 0.05 mg/ml.

Results

- Diagnosed SCC (control) is taken as reference for the present study [Figure 1]
- Leukoplakia [Figure 2]
- OSMF [Figure 3]
- Carcinoma of buccal mucosa [Figure 4].

Summary of the result is tabulated in Table 1. Figure 5 shows the graph of mean difference of DNA doubling.

Table 2 and 3 show the statistical analysis.

Discussion

Several studies have established an independent association of deleterious oral habits such as smoking, chewing tobacco, and betel quid chewing with occurrence of oral cancer.

| Type of cancer | Doubling of DNA count | Colchicine in inhibition of cancer (%) | Diagnosis |
|----------------|-----------------------|----------------------------------------|-----------|
| SCC-9 cell line control | Characteristic feature | 94.90 | Malignant |
| Leukoplakia | Significant | 33.51 | Premalignant |
| Oral submucous fibrosis | Less significant | 31.83 | Premalignant |
| Carcinoma of buccal mucosa | Significantly higher | 63.05 | Malignant |

Results of the 15 oral biopsies were analyzed using Student’s $t$-test. SCC=Squamous cell carcinoma

![Figure 1: Diagnosed squamous cell carcinoma (control) is taken as reference for the present study. Colchicine significantly inhibits the growth of oral squamous cell carcinoma-9 cell line by arresting G2M phase of the cell cycle (94.90%). This cell cycle result indicated that doubling of DNA is a characteristic feature of cancer](image-url)
tobacco and betel quid chewers, have a great prevalence in our country.\[5\]

Both OSMF and leukoplakia have high cancer turnover potentiality; if detected early, it can be prevented and treated effectively. Genotoxicologic studies provide a platform to determine DNA damage in cancer progression. The deleterious oral habits have DNA doubling or DNA damage, which has been clearly established. We have been able to demonstrate in this study that DNA doubling is associated with oral cancer and leukoplakia and also that DNA doubling is associated with deleterious oral habits.

According to Mukherjee et al., DNA damage measured by single-cell gel electrophoresis is greater in leukoplakia and SCC, but not in OSMF. Deleterious oral habits are also associated with greater DNA damage.

In a study done by Udupa et al., there was increase in the tail length in buccal epithelial cells of OSMF group when compared with the healthy group.\[6\] Furthermore, there was a significant increase in the DNA damage with duration of habits.

This study was carried out for early detection and to determine the prognosis of leukoplakia OSMF and OSCC by inhibition of DNA doubling at G2M phase of cell cycle.
cycle by flow cytometry analysis. We had been able to demonstrate that DNA content at G2M phase of cell cycle by flow cytometry analysis is greater in oral cancer and leukoplakia, compared to OSMF. Inhibition of G2M phase of cell cycle by colchicine provides a platform to determine the cancer progression.

**Conclusion**

DNA analysis using colchicine facilitates early detection and also helps determine the prognosis of leukoplakia, OSMF, and oral cancer. It is suggested that further research with larger sample size is necessary. The present study could prove to be an excellent aid for the screening of potentially malignant disorders, which will help in early detection of oral cancer, thereby decreasing morbidity and mortality associated with it.

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**Conflicts of interest**

There are no conflicts of interest.

**References**

1. Mukherjee S, Ray JG, Chaudhuri K. Evaluation of DNA damage in oral precancerous and squamous cell carcinoma patients by single cell gel electrophoresis. Indian J Dent Res 2011;22:735-6.
2. Rabinovitch P. “Introduction to cell cycle analysis.” Available from: http://www.phnxflow.com/Introduction to Cell Cycle Analysis.pdf. [Last accessed on 2017 Feb 08].
3. Paul A, Manjula. Cytotoxic and antiproliferative activity of Indian medicinal plants in cancer cells. Int J Sci Res 2014;3:88-93.
4. Wersto RP, Chrest FJ, Leary JF, Morris C, Stetler-Stevenson MA, Gabrielson E. Doublet discrimination in DNA cell-cycle analysis. Cytometry 2001;46:296-306.
5. Rao J PK. Potentially malignant lesion - Oral leukoplaia. GARJMMS 2012;1:286-91.
6. Udupa R, Hallikeri K, Trivedi DJ. The comet assay a method to measure DNA damage in oral submucous fibrosis patients: A case-control study. Clin Cancer Investig J 2014;3:299-304.