Research Article

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Identification, Evaluation and Comparative Assessment of Apoptotic Cells in Oral Epithelium of Pre Malignant Lesion and Oral Squamous Cell Carcinoma Under Light Microscope

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ABSTRACT

Aim: 1. Identify And Count The Apoptotic Cells In Normal Mucosa, Oral Epithelial Dysplasia And Oral Squamous Cell Carcinoma (OSCC). 2. Compare The AI Between Normal Mucosa, Oral Epithelial Dysplasia And OSCC. 3. Compare The AI With Histological Grading Of Epithelial Dysplasia And OSCC.

Materials and Methods: 50 tissue specimens were collected from patients with oral leukoplakia and OSCC. The biopsy was done and tissue was fixed at 10% buffered formalin, processed for routine paraffin sections, sections taken, stained by Hematoxylin and Eosin and examined under light microscope, using 40× objective and 10× eyepiece. Apoptotic bodies were counted in each high-power field (HPF). Statistical Analysis: Statistical analysis was done by using one way analysis of variance to find the significance of mean apoptotic cell counted among study groups as well as among various grades in dysplasia and OSCC. Post-hoc Tukey test was used to find the pairwise significance between the groups.

Results: It was observed that AI increased from the increasing grade in dysplasia and decreased from the increasing grade in OSCC.

Conclusions: The recently introduced histological parameter of AI can help to predict the survival rate by early diagnosis and early treatment of cancer.

Keywords: Apoptotic count, oral dysplasia, oral squamous cell carcinoma.

INTRODUCTION

Cancer is the second largest cause of human dying worldwide. Cancer has become one of the leading reasons of death in India.[1,2] Most of the oral cancers are squamous cell carcinomas and usually preceded by the pre-existing potentially malignant lesion that could present as persistent leukoplakia or oral submucous fibrosis, and occasionally de novo. [3] Majority of oral cancers are associated with tobacco chewing. The transformation rate of dysplasia to cancer is 36.4%. Assessment and prognosis of the transformation potential depend on the histological grading, which is done by quantifying the degree of architectural and cellular abnormality in the epithelium by H and E stained section. [4, 5]

Stage of progression of OSCC at the time of diagnosis is the most important prognostic factor. Within 5 years of initial diagnosis around half of the patients detected for oral cancer will die. The 5-year survival rate has not improved with the advent of rationally targeted drugs and in spite of a better grasp of molecular level of cancer.[3,5,6]

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Recently histological parameters of cell proliferation and cell death have become important for those individuals who are at a high risk of developing carcinoma. [8] Apoptosis or programmed cell death is a natural process of self-destruction in certain cells. Apoptosis is determined by the genes and can be initiated by a stimulus or by removal of a repressive agent. It is a physiological process that helps to keep the number of cells relatively constant by compensating for mitosis. It is necessary to prevent the tumor formation that means particular cells are programmed to die at specific sites and specific stages of development. [9]

Thus, the present study was done with the purpose of evaluating the apoptotic cell/apoptotic bodies and comparative assessment among oral epithelial dysplasia's (OED) and oral squamous cell carcinoma (OSCC) under a light microscope using routine Haematoxylin and Eosin stain. As the counting of apoptotic bodies using light microscopy is feasible and the technique has been used and described by several authors.

Aim of the study

The aim of the study, was to count the apoptotic cells in oral potentially malignant lesions and oral squamous cell carcinoma to evaluate its prognostic role.

Inclusion criteria:

Clinically and histologically proven cases of oral leukoplakia and oral squamous cell carcinoma cases.

Exclusion criteria: Patients who had previously undergone treatment for oral leukoplakia and oral squamous cell carcinoma and those patients who were having any other systemic diseases such as diabetes mellitus, bleeding disorders, cardiovascular disorders were excluded from the study.

Armamentarium required:

Materials used:

10% Neutral buffer Formalin, Gloves, Bard parker knife and handle, Tissue holds forceps, Tissue holding cassette, Leukart's L blocks, Methyl Alcohol, Xylene, Molten paraffin wax, Section adhesive (Egg albumin), Glass Slide, Cover Slips, H & E stains, DPX Mounting media, Cedar wood oil.

Equipments Used:

Automatic tissue processor, Soft tissue microtome Paraffin water bath with thermostat, Thermostat controlled slide warming table, Binocular light Microscope, C-MOS Camera.

MATERIALS AND METHOD

Patients detailed case history along with the past medical history was taken. Patients written consent was taken and incisional biopsy was performed under local anesthesia from a representative part of the oral mucosa. The tissue was immediately immersed in formaline for fixation. 5µm sections were cut of these formalin fixed paraffin embedded tissues and stained with H and E stain and observed under light microscope using oil immersion high magnification.

Criteria for identification of Apoptotic Cell/ bodies

Apoptotic cell were counted separately in each field and then mean value was obtained. Apoptotic bodies were identified and counted according to the morphological criteria proposed by Kerr et al.[8]

This includes:

1. Cell shrinkage (smaller, eosinophilic cytoplasm with round and smooth margin separating from neighboring cells).

2. Chromatin condensation (hyperbasophilic in color and irregular in shape).

3. Nuclear fragmentation (one or more chromatin pieces, round in shape and vary in size).

4. Non-inflamed field.

Counting of apoptotic cells and calculation of apoptotic index: For calculating the Apoptotic index (AI), from each section, 1000 cells were evaluated for the presence of apoptotic cells. The AI was expressed as a percentage of the total number of non-apoptotic cells counted.

Statistical Analysis

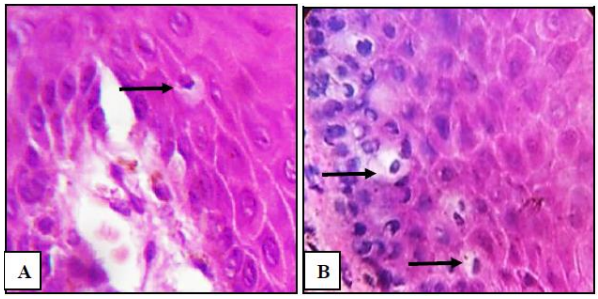

Fig a and b: Histopathological image showing presence of apoptotic cells in epithelial dysplasia. (H&E; X 1000)

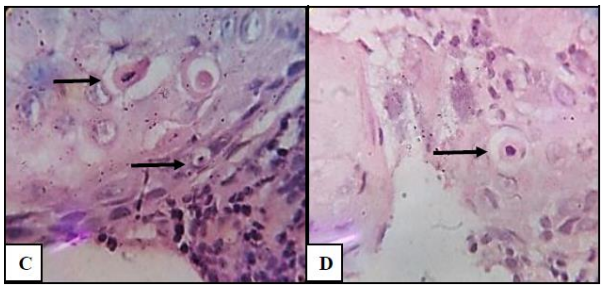

Fig c and d: Figure C and D: H & E stained section showing presence of apoptotic cells in OSCC(H&E; X 1000)

Table 1: Comparison of mean apoptotic index across various study groups.

Groups	N	Apoptotic Index		
		Range	Mean±SD	95%CI
Control	10	0.2 - 1.2	0.76±0.35	0.50 - 1.01
Leukoplakia	20	0.9 - 3.2	1.87±0.61	1.58 - 2.15
OSCC	20	1.3-7.8	3.15±1.65	2.38-3.92
Inference	P<0.001 (HS), F= 16.116			
Pairwise comparison	Control – Leukoplakia= 0.03* Control – OSCC <0.001* Leukoplakia – OSCC= <0.002*			

*Indicated significance at 0.05 levels.The apoptotic index in group A ranged from 0.2 to 1.2 with a mean value of 0.76, standard deviation of 0.35 and a 95% confidence interval of 0.50 -1.01. (Table 1, Graph 1). The Apoptotic index in Group B consisting of cases of Oral leukoplakia ranged from 0.9 to 3.2 with a mean value of 1.87, standard deviation of 0.61 and 95% confidence interval of 1.58 -2.15. (Table 1, Graph 1).

The data obtained was tabulated in Microsoft excel and analyzed in IBM SPSS 23.0 (IBM Corporation). One Way ANOVA was performed to compare mean values of the apoptotic index among different study groups, grades of epithelial dysplasia and grades of

Table 2: Comparison of mean apoptotic index among grades of Epithelial Dysplasia.

Histopathological grade of Epithelial Dysplasia	Range	Mean±SD	95% CI
Mild Epithelial Dysplasia	0.9 - 2.2	1.45±0.42	1.15-1.80
Moderate Epithelial Dysplasia	1.1-2.7	2.07±0.51	1.68-2.46
Severe Epithelial Dysplasia	2.2-3.2	2.70 ±0.71	-3.65-9.05
Inference	P<0.05 (S), F=6.616		
Pairwise comparison	Mild - moderate = 0.049* Mild - severe = 0.013* Moderate - severe= 0.245		

*Indicated significance at 0.05 levels.

Table 3: Comparison of apoptotic cell carcinoma with histopathological grades of OSCC.

Histopathological grade of OSCC	Range	Mean±SD	95% CI
Well Differentiated OSCC	2.1-7.8	4.13±2.12	1.91-6.36
Moderately Differentiated OSCC	2.3-6.1	3.16±1.23	2.28-4.04
Poorly Differentiated OSCC	1.3-2.1	1.65±0.41	0.99-2.31
Inference	P<0.05*, F=3.43		
Pairwise comparison	Well - moderately =0.423 Well - poorly= 0.045* Moderately - poorly =0.220		

*Indicated significance at 0.05 levels.

Apoptotic cell count in well differentiated SCC ranged from 2.1-7.8 with a mean of 4.13 and a standard deviation of 2.12. In moderately differentiated SCC, apoptotic cell count ranged from 2.3-6.1 with a mean of 3.16 and a standard deviation of 1.23 and in cases of poorly differentiated SCC apoptotic cell count ranged from 1.3-2.1 with a mean value of 1.65 and standard deviation of 0.41. (Table 3, Graph 3)

OSCC. 'P' value <0.05 was considered to be statistically significant. Post Hoc Tukey's HSD analysis was used for pairwise comparison between study groups. The statistical difference of the mean apoptotic cell count among the three groups of control, oral leukoplakia and oral squamous cell carcinoma were analyzed. Probability 'p' was calculated. Spearman's non-parametric correlation

was used to establish the correlation between the mean apoptotic index and study groups, grades of epithelial dysplasia and grades of OSCC. Microsoft word and Excel have been used to generate graphs, tables etc.

Table 4: Spearman's Correlation of mean apoptotic cell count with various study groups, grading of epithelial dysplasia and grading of OSCC.

			Mean Apoptotic cell count
Spearman's rho	Study Group	Correlation coefficient	0.743
		Sig. (2 tailed)	0.000*
		N	50
	Grading of epithelial dysplasia	Correlation coefficient	0.648
		Sig. (2 tailed)	0.002*
		N	20
	Grading of OSCC	Correlation coefficient	-0.573
		Sig. (2 tailed)	0.008*
		N	20

* Correlation is significant at the 0.01 level (2-tailed).

RESULTS

In the present comparative study, the apoptotic index was calculated in 3 different groups consisting of 10 controls designated as group A, 20 subjects with oral leukoplakia designated as group B and 20 subjects with oral squamous cell carcinoma designated as Group C. Apoptotic index was calculated in all the 3 groups and the arithmetic means of the apoptotic cell count were calculated. Upon comparison of mean apoptotic index between different study groups, i.e. control, leukoplakia and OSCC the p value was less than 0.001, the statistical difference was found to be highly significant. (Table 1). Pair wise comparison of mean apoptotic index between control & Leukoplakia, Control & OSCC and leukoplakia & OSCC shows that the difference was statistically significant with 'p' values of 0.05, 0.001 and 0.05 respectively.(Table 1). When a comparison of mean apoptotic index among different grades of epithelial dysplasia was made, it shows that mean apoptotic index increases with increase in the grading of dysplasia and the difference was found to be statistically significant with p value of 0.05. Pairwise comparison of mean apoptotic index between mild-moderate and mild to severe epithelial dysplasia showed statistically significant p values, whereas between moderate and severe epithelial dysplasia the p value was found to be non-significant (Table 2). Comparison of mean apoptotic index among various grades of OSCC were also made and the results showed that mean apoptotic index decreases with increasing grades of OSCC and the difference was found to be statistically significant. Pairwise comparison of mean apoptotic index between well differentiated & moderately differentiated and moderately differentiated & poorly differentiated OSCC showed statistically non-significant p values, whereas between well differentiated and poorly differentiated OSCC the p value was found to be significant. (Table 3). Spearman's Correlation was used to find the correlation between the mean apoptotic index and grades of epithelial dysplasia and grades of OSCC. The results showed that apoptosis, cell count is directly proportional to the increasing grades of epithelial dysplasia and increasing grades of OSCC. As we move from control to oral epithelial dysplasia to OSCC, the mean apoptotic index increased. The results also showed that the mean apoptotic index is directly proportional to the increasing grades of epithelial dysplasia and inversely proportional to increasing grades of OSCC, which means that the mean apoptotic index increases with increasing grades of epithelial dysplasia and decreases with increasing grades of OSCC (Table 4).

DISCUSSION

Our study is based on the evaluation of AI in 50 oral leukoplakia and oral squamous cells carcinoma on light microscopy. Apoptotic bodies were counted using ×1000 magnification (under oil immersion). We observed that an accurate assessment of apoptosis is possible by light microscopy. Care must be taken to distinguish lymphocytes from apoptotic bodies and not to include neutrophils or other leukocytes in the count. In our study the apoptotic index increased from oral epithelial dysplasia to oral squamous cell carcinoma. It is similar to the

study by Nalambi Set al. (2016)[10] who concluded that apoptotic index increased from oral epithelial dysplasia to oral squamous cell carcinoma, which was statistically significant. [10]When we compared the AI between grades of oral dysplasia and OSCC, we found that the mean apoptotic index in leukoplakia patients increased progressively with increasing grade and the correlation was found to be statistically significant with p value of 0.05. This is in accordance with the studies conducted by Birchall M (1996)[11] and Jain A et al. (2009)[3]. They concluded that the mean AI increases progressively with increasing grades of dysplasia. However, in the study by Vishvanathan V et al. (2015)[12] AI increased gradually from normal mucosa to leukoplakia but slight drop in AI was seen in severe dysplasia when compared to moderate dysplasia, which was found to be statistically insignificant. Present study also compares mean apoptotic index among various grades of OSCC and the results showed that mean apoptotic index decreases with increasing grades of OSCC and the difference was found to be statistically significant with p value of 0.05. The highest AI was noted in WDSCC. This is in accordance with the studies conducted by Birchall M (1996)[11], Jain A et al. (2009)[3] and Vishvanathan V et al. (2015)[12] they concluded that the mean AI increases progressively with increasing grades of dysplasia, with the maximum AI in well-differentiated (WD) squamous cell carcinoma and a fall was noted with progression toward higher grades of OSCC.[3, 13] Hence, we can conclude that a low AI suggests aggressive behavior of tumor tissue. The results showed that mean apoptotic index is directly proportional to grading of epithelial dysplasia and inversely proportional to grading of OSCC. We observed that an accurate assessment of apoptosis is possible by light microscopy. Apoptotic bodies were seen in the suprabasal and basal regions of the normal oral mucosa and early dysplastic lesions but as the severity of the premalignant and malignant lesion increases, the apoptosis becomes generalized. It is similar to the study by Vishvanathan V et al. (2015).[12] Tumor growth is a summation of mitosis and cell production and cell loss and death. Thus, a high AI in WD SCC as observed by us possibly suggests that the tumors that exhibit more apoptosis may be slower growing and therefore may be less biologically aggressive as suggested by Saini R et al (2010).[13]An association of low AI with increasing of OSCC can be an indication of poor prognosis. Although there appear to be valid biological reasons for a relationship between low AI and poor prognosis, it is likely that other factors in tumor progression such as mitotic rate and invasive capability have a confounding influence on tumor behavior than the AI alone. Numerous stimuli can induce apoptosis in a cell type-dependent manner. Depending on the triggering factor and the cell type, there are multiple signaling pathways that lead to activation of the apoptotic machinery. Change in the control of apoptosis is a critical step in the development of invasive change in a premalignant tissue. [14] Chromosomal aberrations and accumulation of mutations in many genes encoding crucial proteins or oncoproteins that control cell growth and apoptosis may also induce neoplastic transformation.[50] Analysis of AI showed a progressive increase in apoptosis in parallel with biological aggressiveness, indicating increased synthetic activity of proteins during neoplastic progression. As the tumor grows, there is an increase in cell proliferation, and probably, due to the large tumor size and a high growth rate potential, the tumor outgrows its blood supply leading to hypoxic injury-causing increased apoptosis.[15,16] Increased apoptosis with increasing grades of neoplastic lesions is associated with large tumor size and with a shortened disease-free survival period. Compelling evidence indicates that oncogenic changes promote apoptosis during multistage carcinogenesis. [17, 18] The ability to predict which tumor cells will survive to grow as metastases may be useful in predicting the outcome. The beneficial anticancer effects of chemotherapy are predominantly mediated through induction of apoptosis in tumor cells de novo or as a result of chemotherapy-induced damage of cellular metabolic processes or cell cycle control mechanisms. Thus, it is possible that tumors that exhibit apoptosis may be more sensitive to chemotherapy and hence likely to have a better prognosis.[14]

CONCLUSION

From the observations we gathered in the present study on apoptotic cell count in oral leukoplakia, oral squamous cell carcinoma and age- and sex-matched controls; we have arrived at the following conclusions:

1. When the patient's age & sex attributes were considered, the highest incidence of oral leukoplakia was observed in the 3rd and 4th decade with male preponderance and in the 4th& 6th decade & males outnumbered females in relation to OSCC.

2. Definitive association was found between the harmful habits of tobacco chewing combined with smoking & alcohol consumption to the incidence of the disease.

3. Mean Apoptotic index was increased significantly in oral leukoplakia and OSCC compared to controls.

4. From the present study it was also concluded that on mean apoptotic index increased significantly with increasing grades of epithelial dysplasia.

5. It was also evident that mean apoptotic index decreased significantly with increase in histopathological grading of OSCC. Oral cancers that exhibit less apoptosis tend to show aggressive behavior and have a greater potential for metastasis. From this histological evaluation, we can conclude that AI increased with increasing severity of the lesion. Decrease in the AI with increasing grades of carcinoma could act as a poor prognostic indicator. AI would act as a better marker for the disease progression. Thus, we emphasize that the histopathology report of every premalignant and malignant squamous cell lesions of the oral cavity should include AI with histomorphology and such studies should be performed on a larger sample size inclusive of all age group, races and genders. This would help in timely surgical intervention and will result in less deformity and a better outcome prognostically. Further, studies need to be undertaken to detect and understand the apoptotic mechanisms in the progression from OED to OSCC.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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