CELL PROLIFERATION IN ORAL SQUAMOUS CELL CARCINOMA IN DIFFERENT SUBSITES

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partial fulfillment of the requirements
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BRANCH – VI
ORAL PATHOLOGY AND MICROBIOLOGY

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Chennai – 600 032

2010 - 2013

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled, "CELL PROLIFERATION IN ORAL SQUAMOUS CELL CARCINOMA IN DIFFERENT SUBSITES", is a bonafide and original research work done under supervisor **Dr. I. Ponniah,** Professor and head, Department of Oral Pathology, TamilNadu Government Dental College and Hospital belong to **Dr.Arya.A.N,** post graduate student, Department of Oral Pathology, TamilNadu Government Dental College and Hospital, Chennai – 600 003.

I also firmly state that I, **Dr.Arya.A.N** is entirely responsible for any ethical violations (if any) and it does not have any binding on my supervisor.

Name of the Candidate : **Dr.Arya.A.N**

Signature of the Candidate :

CERTIFICATE

This is to certify that **Dr.ARYA.A.N**, Post Graduate student (2010-2013) in the Department of Oral Pathology and Microbiology, Tamil Nadu Government Dental College and Hospital, Chennai - 600 003, has done this dissertation titled **"CELL PROLIFERATION IN ORAL SQUAMOUS CELL CARCINOMA IN DIFFERENT SUBSITES**" under my supervision in partial fulfillment of the regulations laid down by the **Tamil Nadu Dr.M.G.R. Medical University**, Chennai-600 032 for **M.D.S.,** (Branch-VI) **Oral Pathology and Microbiology** degree examination.

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PRINCIPAL

Tamil Nadu Government Dental College and Hospital, Chennai - 600 003

TRIPARTITE AGREEMENT

This agreement herein after the "Agreement" is entered into on this day " ---------- between the Tamil Nadu Government Dental College and Hospital represented by its **Principal** having address at Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, (hereafter referred to as, 'the college')

And

Dr. I. Ponniah aged 45 years working as **Professor and Head** in Department of Oral Pathology at the college, (herein after referred to as the 'Co-Investigator')

And

Dr. Arya.A.N aged 27 years currently studying as **Post Graduate student** in Department of Oral Pathology, Tamil Nadu Government Dental College and Hospital, Chennai – 600 003, (hereafter referred to as 'the PG student and principal investigator')

Whereas the PG student as part of her curriculum undertakes to research on "CELL PROLIFERATION IN ORAL SQUAMOUS CELL CARCINOMA IN DIFFERENT SUBSITES" for which purpose the Co-Investigator and the college shall provide the requisite infrastructure based on availability and also provide facility to the PG student as to the extent possible as a principal investigator

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Witness **Co-investigator and Supervisor**

1.

2.

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ABSTRACT

BACKGROUND:

Cell proliferation rate is one of the important determinants of prognosis in cancer. Oral cancer prognosis differs among different subsites and it is also influenced by the etiological factors.

AIM AND OBJECTIVE:

To compare the cell proliferative index and to evaluate the role of causative factors among different sub sites of patients with oral squamous cell carcinoma by assessing the cell proliferation by mean AgNOR counts.

MATERIALS AND METHODS:

This is a prospective study and a total of 94 subjects with histologically proven oral squamous cell carcinoma were included. They were classified into four groups based on the site of the lesion, namely, carcinoma of buccal mucosa (group I), carcinoma of alveolar mucosa (group II), carcinoma of tongue (group III), carcinoma of retromolar trigone area (group IV). Each group was inturn subdivided into sub-groups based on the etiological factors. The etiological factors considered in our study were betel quid chewing, mawa or gutka chewing and smoking for group I, group II and group IV and trauma was considered to be an etiological factor for group III. The cell proliferation marker used in this study are the silver-stained nucleolar organizer regions (AgNORs) and mean AgNOR count was used to compare the cell proliferation rate among the four groups and amongst the sub-groups in each group. The results were analysed for statistical significance.

RESULTS:

The mean AgNOR count for group III (carcinoma of tongue) was significantly higher than the other groups. Amongst the sub-groups of group I (carcinoma of buccal mucosa), sub-group with etiology of smoking showed significantly higher mean AgNOR counts. In case of group III (carcinoma of tongue) sub-group with trauma as the etiological factor showed significantly higher mean AgNOR counts than sub-groups with other etiological factors

CONCLUSION:

Carcinoma of tongue showed greater cell proliferation rate when compared to other subsites of oral cavity considered in this study. Thus greater cell proliferation rate could be one of the reasons for poor prognosis of tongue cancer as established by many other studies. Trauma causes greater cell proliferation rate when compared to other etiological factors in tongue. In buccal mucosa, smoking causes greater cell proliferation when compared to other etiologies. Further studies are required to establish the influence of the site of origin and different etiological factors on cell proliferation rate and hence the prognosis of oral squamous cell carcinomas.

Key words: Cell proliferation, Oral cancer sub-sites, AgNORs,

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ABBREVIATIONS

OSCC	Oral Squamous Cell Carcinoma
NORs	Nucleolar Organiser Regions
AgNORs	Argyrophilic Nucleolar Organiser Regions
mAgNOR	Mean AgNOR count
SG	Sub-group
PCNA	Proliferating Cell Nuclear Antigen
P value	Probability value

INTRODUCTION

The squamous cell carcinoma is the most common malignant neoplasm of the oral cavity accounting to about 85% of the total oral malignant neoplasms.[1] Oral squamous cell carcinoma (OSCC) is the eighth most common cancer worldwide, but the incidence is higher in developing countries when compared to developed countries. The age-standardised mortality rate due to oral cancer in India is greater than 3.0 per 100,000 populations which is very high compared to other areas.[2]

The prognosis of oral squamous cell carcinoma depends on multiple factors, of which cell proliferation is one of the most important factors.[3,4] The proliferative activity of the cell depends on the cell cycle which is inversely proportional to the speed of the cell cycle or the generation time, and directly proportional to the proportion of cells committed to enter the cell cycle or the growth fraction.[4] However, unlike the normal cells, proliferation of neoplastic cells occurs in the absence of corresponding increase in the epithelial cell loss.[5]

The proliferative activity can be assessed by cell proliferation markers, which are classifiable as growth fraction markers, markers of specific phases of cell cycle and cell cycle time markers. The growth fraction can be determined by MIB1 or Ki-67 antibodies which identify the antigen expressed in non-mitotic phases (G1, S and G2) of the cell cycle.[6] The mitotic phase can be evaluated by counting the mitotic figures which is an oldest and popular way of assessing the cell proliferation.[7] The S phase fraction can be assessed by incorporation techniques which use titrated thymidine (TH3) or bromodeoxyuridine and by immunohistochemical assessment for proliferating cell nuclear antigen (PCNA), a nuclear protein involved in DNA

synthesis or cyclins.[6] The percentage of cells in various phases of cell cycle can also be determined by flow cytometry.[8]

The cell cycle time can be evaluated by simpler procedures like quantification of the argyrophilic proteins associated with the nucleolar organizer regions (AgNORs).[8] AgNOR techniques mark the proteins associated to the nucleolar organizer regions (NORs). NORs are loops of DNA that transcribe for ribosomal RNA. They are located on the short arm of chromosomes 13, 14, 15, 21, and 22. There are certain acidic and argyrophilic, nonhistonic proteins called NOR-associated protein codes in these regions. NORs can be demonstrated in tissue sections by staining their associated proteins with colloidal silver and these silver stained reaction products represent the AgNOR.[9] According to Sirri et al, 2000,[10] the higher the number of NORs, the lower is the duration of the cell cycle and the higher the rate of cell proliferation. Therefore, the quantative analysis of AgNOR is an excellent indicator of cell proliferation that may predict the prognosis of tumors.[9,10,11] Also, AgNOR is cheaper, reliable and less time consuming when compared to other methods[11] and the results obtained are comparable with other methods.[12]

Smoking amounts to about 42% of death due to oral cancer worldwide. However in India and its neighbouring countries use of various forms of smokeless tobacco and betel quid with or without tobacco are the major risk factors.[13] In the population under study, smoking, use of smokeless tobacco in the form of maawa, gutka and snuff dipping, betel quid chewing and trauma were found to be the common etiological factors. But each of these causative factors has been known to differentially influence the cell proliferation rates in the oral mucosa.[14,15,16]

Although the prognosis of OSCC differs among sites within the oral mucosa,[17,18] it is not clear whether the cell proliferation is in itself a prognostic determinant, especially among different subsites when exposed to different causative factors.

The purpose of this study is t**o compare the cell proliferative index and to evaluate the roles of causative factors among subsites within the oral cavity of patients with oral squamous cell carcinoma** by **assessing the cell proliferation by (mean) AgNOR counts.**

AIM

To compare the cell proliferative index and to evaluate the role of causative factors among different sub sites of patients with oral squamous cell carcinoma.

OBJECTIVE

To assess the cell proliferation by mean AgNOR counts in oral squamous cell carcinoma.

REVIEW OF LITERATURE

CELL PROLIFERATION AND CANCER

Pardee AB et al,[19] ***in 1989*** suggested that cells that enter G1 phase are the main determinants of the cell proliferation rate and are defectively controlled in cancer cells.

Ames BN et al,[20] ***in 1990*** attempted to clarify the mechanism of carcinogenesis. In their perspective they have stated that a dividing cell is much more at risk of mutating than a resting cell and many stable mutations can occur during cell division due to endogenous mutagens which form oxidative products which inturn causes massive damage to DNA. This oxidative damage is the major contributor to degenerative changes leading to cancer. Thus any agent causing chronic mitogenesis is mutagenic and they concluded that mitogenesis increases mutagenesis.

Cohen SM et al,[21]***in 1990*** illustrated the critical role of cell proliferation in carcinogenesis using two protypical compounds, a genotoxic carcinogen 2-acetylaminofluorene (2-AAF), and a nongenotoxic agent, sodium saccharin. They suggested that the carcinogenic dose-response relationship for these genotoxic chemicals was also due in part to increased cell proliferation. Mechanistic information is required for determination of the existence of a threshold for the proliferative (and carcinogenic) response of nongenotoxic chemicals and the estimation of risk for human exposure.

Weinstein IB[22] ***in 1991*** contradicted the the theory that that mitogenesis is the major rate-limiting factor in carcinogenesis requires that cell replication per se be highly hazardous because of the inherent danger of spontaneous mutations. He

believed that cell replication is one of the roles (but not the only role) of carcinogenic agents

Croy RG[23] ***in 1993*** examined the current understanding of the mechanisms by which chemicals provoke cell proliferation and the contribution of various kinetic patterns of cell proliferation to carcinogenesis. In this review, he insisted that cell division plays a key role at each stage in the evolution of cancer, and it is well documented that increased rates of cell proliferation can escalate the risk of malignancy.

Thompson PJ et al,[24] ***in 2002*** studied the relationship between epithelial cell proliferative activity and oral cancer progression. Archival tissue specimens from 10 previously treated patients with oral cancer with 3-years follow up were evaluated for cell proliferation markers like Ki67, cyclin A and histone mRNA cell cycle markers. While histone mRNA labelling ultimately proved unreliable, both Ki67 and cyclin A labeling indices demonstrated an enhanced labelling to occur in increasingly dysplastic and neoplastic tissue. They also showed increase ki67 and cyclin A labeling indices and suprabasal labeling in patients who developed recurrence of the lesion or lymph node involvement thus indicating poor prognosis. Thus the measurement of cell proliferative activity in individual oral epithelial dysplastic lesions or invasive squamous cell carcinomas can provide predictive information on clinical outcome.

Preston-Martin et al[16] ***in 1990***, dicussed examples of human cancer in which increased cell division leads to neoplastic transformation due to accumulation of genetic defects. He had discussed about many risk factors causing cell proliferation pertaining to the site of the lesion. He had discussed tobacco as one of the chemical

agents causing increased cell proliferation in the oral cavity. He also added that all quids that are usually held in buccal mucosa are a source of mechanical trauma and those that contain salked lime have a caustic effect which in turn increases cell proliferation to replace lost cells.

CELL PROLIFERATION MARKERS

van Diest PJ et al,*[4] *in 1998 in his review aimed to provide an overview of methods currently available for assessment of proliferation, and to discuss critically their cell biological framework, their methodology, and some of the most important applications of these methods. He had described the following methods of assessment of cell proliferation which includes, incorporation techniques using incorporation of labelled nucleotide analogues, such as tritiated thymidine or bromodeoxyuridine (BrdU)., counting of mitotic figures, DNA cytometric analysis of percentage of cells in S phase of cell cycle, immunohistochemical analysis of proliferation associated antigens like PCNA, Ki67, MIB-1 and assessment of AgNORs.

Iatropoulos MJ et al,*[25] *in 1996 discussed the proliferation markers and tabulated the six commonly used proliferation markers which included PCNA, p53, Ki67, AgNORs, statins and thymidine analogues. He also compared PCNA and BrdU markers from 3 tissues, i.e. liver, glandular stomach, and uterus, across 2 or 3 strains of rats. He concluded that that PCNA is the most reliable and versatile of all markers used, capable of rendering good results even from archival specimens.

Liu SC et al,*[26] *in 2000 have reviewed the recent literature on immunohistochemical markers of cell proliferation in normal oral epithelia and leukoplakias. Most findings, pointed to an increased proliferation in oral leukoplakias that correlates with the degree of dysplasia. These changes were detected with several

markers including PCNA, Ki-67 (Mib-1), cyclin D1 and CENP-F as well as with procedures using pulse labeling with BrDU, IrDU and tritiated thymidine. Comparison of all methods showed more similarities than discrepancies.

Lindboe CF et al,[7] ***in 2002*** compared the Ki67 equivalent antibodies with regard to qualitative and quantitative immunohistochemical staining characteristics. He compared the staining characteristics of monoclonal MIB-1, monoclonal MM1, polyclonal NCL-Ki-67p, polyclonal Rah Ki-67. The MIB-1 antibody appears to have a higher sensitivity for detecting the Ki-67 antigen than the other three tested antibodies.

Dissanayake U et al,[27] ***in 2003*** studied the cell proliferation stautus in oral squamous cell carcinomas by comparing the cell proliferation rate using Ki67 index in the centre and advancing front of the tumour. The Ki67 index was significantly higher in the advancing front when compared to the centre of the tumor which indicates that the cells in the invasive front are more proliferating and hence they suggested that it is likely to be more informative in cell cycle studies and in studies involving cell proliferation as prognostic indicator.

The AgNORs

The nucleoli usually disappear during the mitotic phase of cell division. However, at the end of telophase and in interphase they reform round weakly stained chromatin regions which correspond to secondary constrictions of metaphase chromosomes of eukaryotic cells. These regions are called as the nucleolar organizer regions (NORs) and they contain genes that code for ribosomal RNA. The nucleolar organizer regions (NORs) were first described by Heitz in 1931.[9]

Goodpasture C and Bloom SE*[28] *in 1975 attempted to visualize NORs in mammalian chromosomes using silver staining and in-situ hybridization and described silver-stained NORs as Ag-NORs which appeared as black-spherical bodies on yellow-brown chromosomal arms. These represent the chromosomal locations of genes coding for 18S-28S ribosomal RNA. They also suggested that there are various chromosomal proteins associated with NORs called NOR specific proteins which indeed take up the silver stain rather than rRNA itself. These argyrophilic proteins were found to be protein C23 nucleolin and protein B23.[10]

Miller OJ et al*[29] *in 1976 localized nucleolar organizer activity to chromosomes 13, 14, 15, 21(rarely) and 22 in humans by silver-staining method.

Crocker J et al*[30] *in 1989 suggested that AgNORs may present in the following three types of configuration in normal and neoplastic cells. In the first type, the NORs are fully aggregated to form a single rounded dark staining structure with no subdots which corresponds to the nucleolus. This type is commonly seen in resting lymphocytes and quiescent cells. In the seond type, the subsidiary dots can be visualized inside the nucleolus. This type is seen commonly in proliferating cells. The third type comprises of small "true" AgNORs scattered throughout the nucleoplasm which are frequently observed in highly malignant cells. All these features were very evident in cytological sections and carefully prepared paraffin sections.

REVIEW ON AgNOR STAINING AND QUANTITATION

Bloom SE and Good Pasture C*[31] *in 1976 demonstrated a simplified and standardized technique for staining of nucleolar organizer regions in human chromosomes. It was based on ammonical silver nitrate technique by Howell et al, 1975.

Trere D[32] ***in 2000*** critically evaluated various methods that were commonly employed to stain AgNORs in cytopathology and histopathology. He has comprehensively described various methods for AgNOR staining which included Ploton's one-step staining procedure (1989) where the staining was performed at lower temperature when compared the the original method of staining by Howell. He has also described in detail a standardized method proposed by Aubele et al in 1994 in International committeefor AgNOR quantitation, in which they had proposed different staining methods for cytological smears, frozen sections and histological samples fixed in ethanol and formalin. Although the committee had recommended the use of image analysis for counting purpose 84.6% of papers published since 1987 used routine counting method only.

Crocker J et al,[30] ***in 1989*** proposed a standardized method for counting AgNORs. He proposed a method in which first all the silver stained structures are counted, but when lying in groups each cluster is treated as one structure. Then, whereAgNORs can be visualized in the nucleolus, each AgNOR should be counted as a unit together with smaller AgNORs seen outside the nucleolus. However in resting cells it is not possible to resolve separate AgNORs within the nucleoli where they are wholly aggregated. So they suggested that in order to get total AgNOR count both extra-nuclear and intra-nuclear dots should be enumerated.

Derenzini M et al,[33] ***in 1991*** attempted to standardize interphase AgNOR measurement by means of automated image analysis system and used lymphocytes as internal control for the standardization procedure. He also discovered that AgNOR area was influenced by the fixatives used and also the staining time.

Bukhari MH et al,[34] ***2007*** proposed a modified method of AgNOR counting in which they reduced the staining time and used 10% sodium thiosulphate and 1%

gold chloride solution as toning solution. Gold chloride produced better clarity when compared to sodium thiosulphate. They also added that the use of counterstain like neutral red interfered with staining quality and hence did not recommend it. .

SIGNIFICANCE OF AgNOR AS CELL PROLIFERATION MARKER

Trere D et al,[35] ***in 1989*** studied the relationship between interphasic silver-stained proteins of the nucleolar organizer regions (Ag-NOR proteins) and cell replication rate in 13 established neuroblastoma cell lines and used automated image analyser to measure the quantity of Ag-NOR proteins. The results indicated that the amount of Ag-NOR proteins is strictly proportional to the proliferative activity of the cells and hence they suggested its use as a parameter for determining the cell proliferation rate.

Sirri V et al,[10] ***in 2000*** determined the variation of expression of AgNOR proteins in different phases of cell cycle. They quantified the AgNOR proteins in different phases of cell cycle using electrophoresis and western blot analysis. They determined that the amount of AgNOR proteins increased during S-G1 phase and higher the amount of AgNOR proteins signifies greater number of cells in S-G1 phase of cell cycle. Thus AgNORs can be reliably used as cell proliferation markers.

Costa ALL et al,[12] ***in 1999*** compared the effectiveness of AgNOR staining with other proliferative markers like Ki67, PCNA using double-staining technique. The slides were first stained for PCNA and Ki67 immunohistochemical markers separately and then the sections were overstained for AgNOR. They found that there was positive correlation between presence of AgNORs and cells that have taken up PCNA or Ki67 staining. Thus AgNOR is an equally effective marker when compared to PCNA or Ki67.

AgNORs IN ORAL SQUAMOUS CELL CARCINOMA

Xie X et al,[36] ***in 1997*** evaluated the AgNOR counts in normal epithelium, dysplastic epithelium and squamous cell carcinoma of oral cavity. They also tested the AgNOR counts for prognostic significance using clinical parameters. The mean AgNOR counts were significantly higher in squamous cell carcinoma when compared to normal and dysplastic epithelium. Also, logistic rank test revealed cases with mean AgNOR counts greater than 6.2 showed significantly greater recurrence rate. Also greater the percentage of nuclei with more than one AgNORs greater was the tendency for recurrence.

Pillai KR et al,[11] ***in 2005*** analysed the prognostic significance of AgNORs in oral carcinomas. They concluded that mean AgNOR count greater than 2.8 concurred with poor prognosis in both univariate and multivariate analysis. Along with AgNOR counts, the T-status of disease was also found to be an independent predictor for treatment outcome in multivariate analysis. Thus T3 and T4 tumours, with mean AgNOR counts more than 2.8, were deemed to be aggressive and may exhibit resistance to current treatment protocols.

Ashraf MJ et al,[37] ***in 2010*** studied the mean AgNOR counts, proliferative index and graded the variation in size and dispersion of AgNOR dots in cells in normal, dysplastic, primary and metastatic squamous cell carcinoma. The Ki67 percentage is significantly increased from normal squamous to SCC group, and the reactivity of staining were related to histological differentiation. The mAgNOR counts were high in all the cases of primary and metastatic SCC and low in normal squamous tissue and increased in dysplastic lesions.

Chandak AR et al,[38] ***in 2011*** examined the possible association between epithelial proliferation and disease progression in the oral mucosa using the actual proliferation index which is measured by the product of Ki67 score and quantity of AgNOR. There was a significant correlation of Bryne's histological malignancy grading with the argyrophilic nucleolar organizer region count and the Ki-67 labeling index. The actual proliferation index is not only useful as a prognostic factor, but could also be a promising treatment determining modality for patients with premalignant and malignant lesions.

Mekhri S et al,[39] ***in 2010*** carried out a study to analyze the distribution of the AgNOR in oral leukoplakia and oral squamous cell carcinoma, and in their various histological grades, and to assess if the AgNOR distribution could give information on the malignant potentiality in premalignant lesions and aggressiveness of the malignant lesions. The mean AgNOR count was higher in cases of oral squamous cell carcinoma when compared to cases of oral leukoplakia, and the AgNOR counts increased with the increase in the grades of dysplasia indicating a higher proliferative rate with increase in dysplasia

SIGNIFICANCE OF ETIOLOGICAL FACTORS IN ORAL CANCER

Kaur J et al,[40] ***in 1994*** studied the expression of p53 tumor-suppresor gene (a commonly identified mutated gene in diverse types of human cancer and plays an important role in regulation of normal cell proliferation) in normal mucosa, premalignant lesions and oral squamous cell carcinoma from Indian patients who consumed betel, areca nut and/or tobacco. There was higher frequency of p53 overexpression in premalignant and malignant lesions in patients who were heavy consumers of betel, areca nut and tobacco

Lewin F et al,[41] ***in 1998*** conducted a study to identify the possible factors involved in etiology of cancer of head and neck among men in two different geographical location in Sweden. The effects of tobacco smoking, oral snuff and alcohol were investigated. They concluded that there was a dose dependent excess risk of cancer of the head and neck from tobacco smoking whereas there was no significant increase in relative risk for the use of Swedish oral snuff. In case of alcohol, moderate alcohol consumption showed no increase risk among ex-smokers or non-smokers, but an increased risk for oral cancer among current smokers.

.***Znaor et al,***[42] ***in 2003*** assessed the independent and combined effects of different patterns of smoking, betel quid chewing and alcohol drinking in oral, pharyngeal and esophageal cancers. Betel quid chewers with or without tobacco showed the highest risk for cancer in other sites of oral cavity than tongue, pharynx and esophagus. Whereas, smoking showed lesser risk for cancer in oral cavity when compared to pharynx and esophagus.

Fontes PC et al,[43] ***in 2008*** compared the AgNOR counts in exfoliative cytology of non-lesional tongue between smokers and non-smokers. The results showed significantly greater AgNOR counts in lateral border of tongue in smokers when compared to non-smokers. Thus smoking causes greater cell proliferation in lateral border of tongue even in the absence of clinically discernable lesion

Lin WJ et al,[44] ***in 2011*** conducted a prospective study to investigate the association between oral cancer and etiological factors like smoking, alcohol consumption and betel quid chewing. The study group comprised of patients with oral cancer and the control group comprised of patients without oral cancer. A multivariate logistic regression model for exploring relevant risk factors for oral cancer was

created. The odds ratio was higher for betel quid chewers when compared to smokers and there was no significant risk for only alcohol consumers.

Nair U et al,[45] ***in 2004*** postulated the mechanism of carcinogenesis and genotoxicity by more prevalent betel quid substitutes namely gutka and pan masala. Gutka consists of flavoured and sweetened dry mixture of areca nut, catechu and slaked lime with tobacco. It has been implicated as a cause of oral sub-mucous fibrosis in young patients which ultimately has greater potential for malignant transformation. In this review they also added that mawa, which is similar to gutka in composition has been linked to oral submucous fibrosis, oral and esophageal cancer.

Gupta PC et al,[46] ***in 1998*** conducted a survey in Bhavnagar district in Gujarat for assessing the use of various tobacco products and prevalence of oral submucous fibrosis. They found that areca nut was mostly used in form of mawa, a mixture of tobacco, lime and areca nut and mawa chewers showed high relative risk for oral submucous fibrosis and hence they suggested an increase in incidence of oral cancer among maawa chewers in future

Rahman M et al,[47] ***in 2005*** calculated the population attributable risk for bidi smoking and oral cancer in south Asia. They analysed twelve case-control studies conducted in India, Pakistan and Srilanka. Pooled odds ratio suggested that beedi smoking showed significant association with oral cancer cases in south Asia.

PROGNOSIS OF ORAL SQUAMOUS CELL CARCINOMA OF DIFFERENT SUBSITES

Garzino-Demo P et al,[17] ***in 2006*** analysed the outcome of patients undergoing treatment for oral squamous cell carcinoma in an attempt to identify the prognostic value of several clinicopathological parameters. There was significant difference in

the survival rate depending on the site of origin. Carcinoma of tongue showed lesser three year and five year survival rate when compared to carcinoma of gingival, buccal mucosa, buccal- retomolar trigone and floor of the mouth.

Rusthoven K et al in 2008[18] compared the over all survival and cause specific survival in patients with squamous cell carcinoma of tongue and with other subsites. The five year over all survival and cause specific survival rate was lesser for oral squamous cell carcinoma of tongue compared to other subsites. Thus carcinoma of tongue showed poorer prognosis than carcinoma of other subsites of the oral cavity.

MATERIALS AND METHODS

SOURCE & SELECTION OF CASES:

Out-Patient Department,

Department of Oral Pathology and Microbiology,

Tamil Nadu Government Dental College & Hospital, Chennai.

Patients with histologically proven oral squamous cell carcinoma and their respective paraffin tissue wax blocks has been utilized for the study from the period of January 2012 to July 2012.

STUDY GROUPS:

Grouping based on site of biopsy.

Group 1: Carcinoma of buccal mucosa

Group 2: Carcinoma of Tongue

Group 3: Carcinoma of Alveolar mucosa

Group 4: Carcinoma of Buccal sulcus-Retromolar trigone area

As this is a prospective study, the number of cases in each group varied. Minimum number of cases for each group : 10

SELECTION CRITERIA

Inclusion criteria:

Group 1: BUCCAL MUCOSA

Group 2: TONGUE

1. Should have histologically proven squamous cell carcinoma
2. Patients with known history of maawa/gutka chewing, beetel quid chewing, smoking, trauma.

Group 3: ALVEOLUS

Group 4: RETROMOLAR TRIGONE-BUCCAL SULCUS AREA

Same as above except H/o trauma which is relatively uncommon in these areas.

Exclusion criteria:

Clinical exclusion criteria:

- Patients with clinical diagnosis of oral squamous cell carcinoma but not proven histopathologically
- Patients having more than one etiology included in each group.
- Patients with etiologies other than the above mentioned ones.
- Patients with history of maawa/gutka, betel quid chewing and smoking for a period of less than 6 months.
- Premalignant lesions or conditions

Histological exclusion criteria:

- No evidence of invasive squamous cell component in the given section
- Loss of tissue while sectioning

METHODOLOGY:

1. Following selection of subjects based on inclusion and exclusion criteria, written informed consent (**Appendix 1 & 2**), which was approved by the Institute's Ethical Committee, was obtained from all the subjects selected for the study after explaining the study procedure.

2. H/O trauma and habits related to oral squamous cell carcinoma were recorded for the patients with histologically proven oral squamous cell carcinoma reporting to

the Department of Oral Pathology while the patients come to collect the biopsy report.

3. The paraffin blocks of the corresponding patients were retrieved from the Department archives and 4µm thick tissue sections are to be made using microtome.
4. A total of 94 cases were included in our study based on clinical and histological inclusion and exclusion criteria. There were no cases with history of trauma in group I (carcinoma of buccal mucosa) and no cases with history of betel quid chewing in group III (carcinoma of tongue).

5. Staining for AgNORs:

Modified AgNOR staining method as proposed by Bukhari et al,[34] 2007.

The tissue is deparaffinized in several changes of xylene and descending alcohol concentrations. Rehydration is then performed in several changes of ultrapure distilled water. The tissue is then incubated in acid alcohol (three parts ethanol: two parts acetic acid) for 5 min and then rinsed in ultra pure distilled water several times.

Solution A (2% concentration)

Gelatin powder 500mg

Formic acid 250µl

Deionized water 25ml

Solution B (50% concentration)

Silver nitrate 30 g

Deionized water 60 ml

Working solution : to be prepared just before use

Solution A 1 part

Solution B 2 parts

Toning solution

Sodium thiosulphate 10%

Gold chloride 1%

The prepared solutions were stored in polypropylene containers, so as to avoid leaching of cations like sodium from glass containers into the reagents. These cations result in background silver deposition.

Staining procedure:

The pretreated sections are incubated with silver nitrate solution (working solution) in a dark humidified chamber for 38 min at room temperature 37 °C. The sections are then incubated in 10% sodium thiosulphate or 1% gold chloride solution for 5 minutes. The sections are then washed in distilled water, dehydrated in graded alcohol and then xylene and mounted.

6. AgNOR counting :

- The nuclei stain light yellow and outline of nuclei as well as cells were usually clearly visible.
- The AgNORs are visualized as brown black discrete dots of variable size within the nuclei.
- In each section 100 cells were counted. Two to five fields were evaluated in each section. The first field of vision was subjectively chosen. Subsequent fields were systematically selected roughly proportional to the overall size of the tumor area.
- Areas with necrosis, pronounced inflammation, artificial damage, or pronounced keratinization were disregarded. In each field the counting started

in the upper left square, moving downward. Careful focusing was used to visualize all AgNORs within each nucleus. [48]

- The lymphocytes and normal adjacent non dysplastic epithelia were used as internal control for staining.[33]
- The number of AgNORs in 100 tumor cell nuclei is counted and average is taken as mean AgNOR count (mAgNOR).
- AgNOR counting was performed under 1000x (100x objective x 10x eyepiece) using oil immersion according to criteria proposed by ***Crocker J et al. 1989.***[30]
- Firstly, all silver stained structures should be counted, but when lying in groups each cluster (almost aggregated or partly disaggregated nucleoli) treated as one structure.
- Secondly, where AgNORs can be seen separately within a nucleolus, each AgNOR should be counted as a unit, together with the smaller AgNORs seen outside the nucleolus.

7. Statistical analysis:

a. The mean mAgNOR counts are calculated for each study group and compared.
b. The mean mAgNOR counts are calculated for different etiologies for each group of patients and compared.
c. The mean mAgNOR counts for common etiologies for all four subgroups are calculated according to the subsites involved and compared.

The collected data was analysed with SPSS 16.0 version. To describe about the data descriptive statistics mean, S.D were used. For the multivariate analysis the one way analysis of variance (ANOVA) with Post-hoc test Tukey's HSD was used to find the significance difference between the inter group comparison . In all the above statistical tools the probability value **P=0.05** is considered as significant level.

Table 1. MASTER CHART FOR GROUP I

SNo	HP No.	ETIOLOGY
1.	14150	Betel quid chewing
2.	14312	Betel quid chewing
3.	14318	Betel quid chewing
4.	14327	Betel quid chewing
5.	14350	Betel quid chewing
6.	14473	Betel quid chewing
7.	14501	Betel quid chewing
8.	14700	Betel quid chewing
9.	14248	Maawa/gutka chewing
10.	14250	Maawa/gutka chewing
11.	14257	Maawa/gutka chewing
12.	14302	Maawa/gutka chewing
13.	14336	Maawa/gutka chewing
14.	14399	Maawa/gutka chewing
15.	14556	Maawa/gutka chewing
16.	14633	Maawa/gutka chewing
17.	14639	Maawa/gutka chewing
18.	14662	Maawa/gutka chewing
19.	14680	Maawa/gutka chewing
20.	14687	Maawa/gutka chewing
21.	14697	Maawa/gutka chewing
22.	14708	Maawa/gutka chewing
23.	14711	Maawa/gutka chewing

24.	14551	Smoking
25.	14615	Smoking
26.	14641	Smoking
27.	14676	Smoking
28.	14677	Smoking
29.	14690	Smoking
30.	14727	Smoking

SUB-GROUP I A : Etiology of betel quid chewing

SUB-GROUP I B : Etiology of maawa or gutka chewing

SUB-GROUP I C : Etiology of smoking

Table 2. MASTER CHART FOR GROUP II

SNo	HP No	ETIOLOGY
1.	14300	Betel quid chewing
2.	14329	Betel quid chewing
3.	14371	Betel quid chewing
4.	14439	Betel quid chewing
5.	14444	Betel quid chewing
6.	14458	Betel quid chewing
7.	14470	Betel quid chewing
8.	14472	Betel quid chewing
9.	14507	Betel quid chewing
10.	14522	Betel quid chewing

11.	14572	Betel quid chewing
12.	14630	Betel quid chewing
13.	14723	Betel quid chewing
14.	14384	Maawa/gutka chewing
15.	14560	Maawa/gutka chewing
16.	14561	Maawa/gutka chewing
17.	14591	Maawa/gutka chewing
18.	14628	Maawa/gutka chewing
19.	14675	Maawa/gutka chewing
20.	14383	Smoking
21.	14440	Smoking
22.	14559	Smoking
23.	14597	Smoking
24.	14603	Smoking

SUB-GROUP IIA : Etiology of Betel quid chewing habit

SUB-GROUP IIB : Etiology of maawa/gutka chewing habit

SUB-GROUP IIC : Etiology of smoking

Table 3. MASTER CHART FOR GROUP III

SNo	HP No	ETIOLOGY
1.	14265	Maawa/gutka chewing
2.	14359	Maawa/gutka chewing
3.	14421	Maawa/gutka chewing
4.	14477	Maawa/gutka chewing

5.	14645	Maawa/gutka chewing
6.	14671	Maawa/gutka chewing
7.	14692	Maawa/gutka chewing
8.	14726	Maawa/gutka chewing
9.	14151	Trauma
10.	14347	Trauma
11.	14386	Trauma
12.	14468	Trauma
13.	14521	Trauma
14.	14528	Trauma
15.	14598	Trauma
16.	14606	Trauma
17.	14622	Trauma
18.	14661	Trauma
19.	14669	Trauma
20.	14702	Trauma
21.	14736	Trauma
22.	14398	Smoking
23.	14410	Smoking
24.	14704	Smoking
25.	14707	Smoking

SUB-GROUP IIIA : Etiology of maawa/gutka chewing

SUB-GROUP IIIB : Etiology of trauma

SUB-GROUP IIIC : Etiology of smoking

Table 4. MASTER CHART FOR GROUP IV

SNo	HP No	ETIOLOGY
1.	14375	Betel quid chewing
2.	14497	Betel quid chewing
3.	14523	Betel quid chewing
4.	14636	Betel quid chewing
5.	14667	Betel quid chewing
6.	14280	Maawa/gutka chewing
7.	14288	Maawa/gutka chewing
8.	14328	Maawa/gutka chewing
9.	14395	Maawa/gutka chewing
10.	14460	Maawa/gutka chewing
11.	14461	Maawa/gutka chewing
12.	14563	Maawa/gutka chewing
13.	14706	Maawa/gutka chewing
14.	14441	Smoking
15.	14446	Smoking

SUB-GROUP IVA : Etiology of Betel quid chewing

SUB-GROUP IVB : Etiology of Maawa/gutka chewing

SUB-GROUP IVC : Etiology of smoking

Fig 1. Staining kit

Fig 2. Staining solutions

Fig 3. Other Armamentarium

Fig 4. Microscope and Stained slides

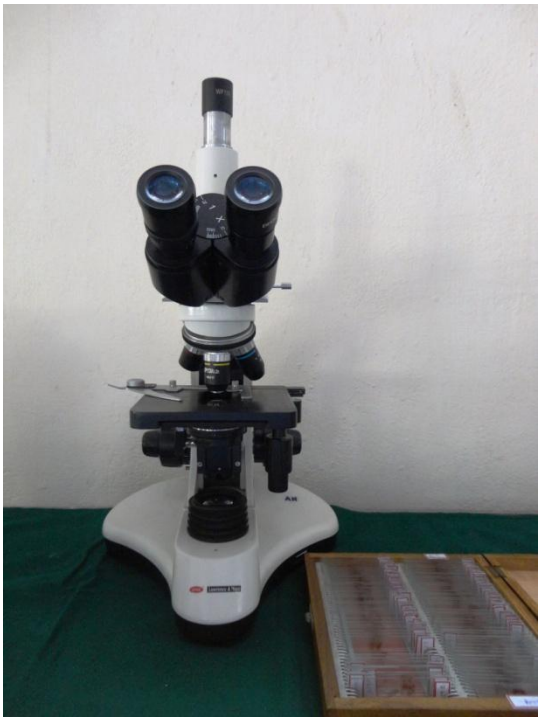


Fig 5. AgNORs (x 400)

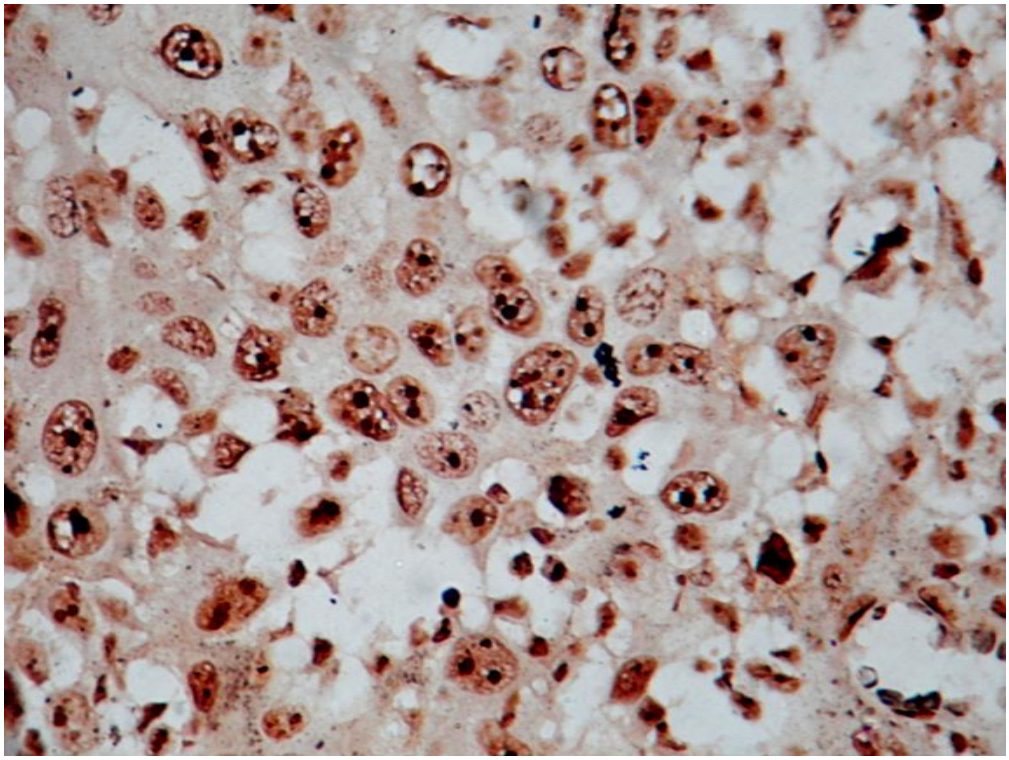


Fig 6. AgNORs (x1000) (oil immersion)

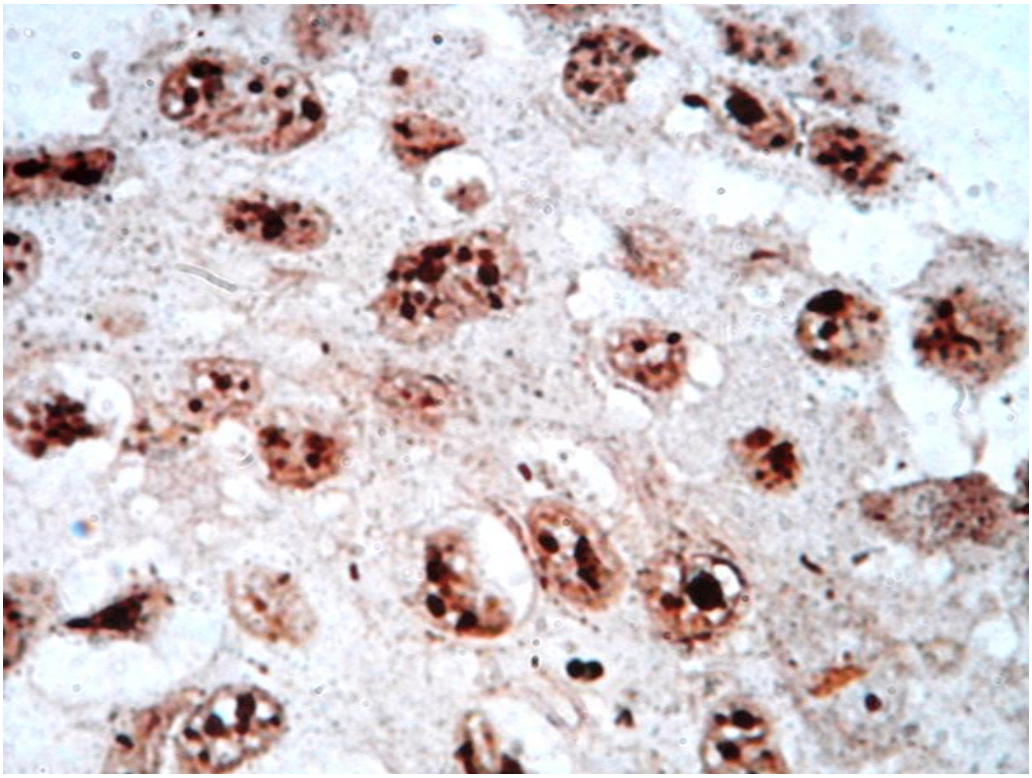


Fig 7. AgNORs in lymphocytes (x 1000) (oil immersion)

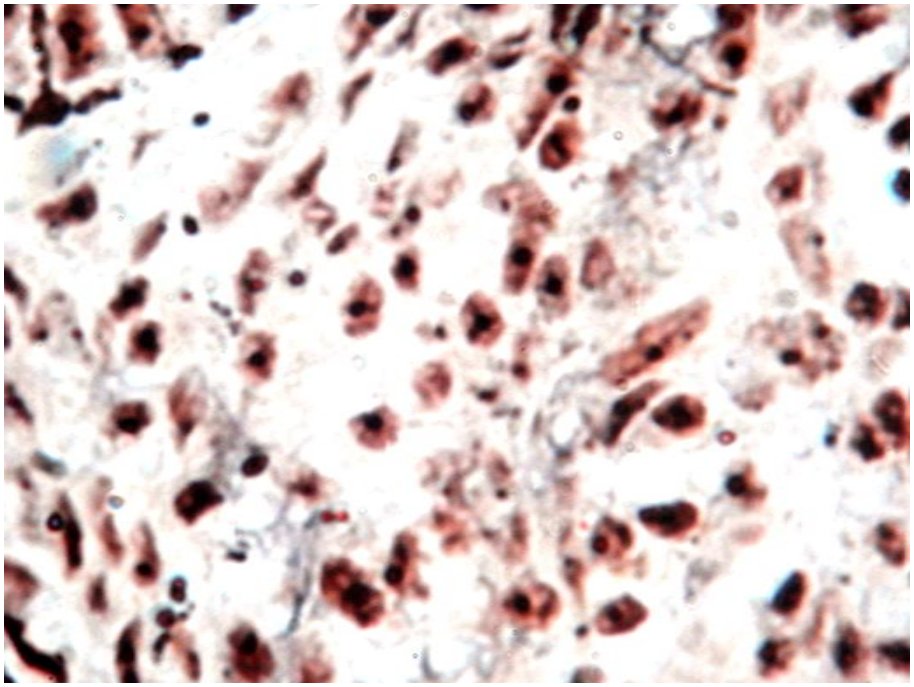
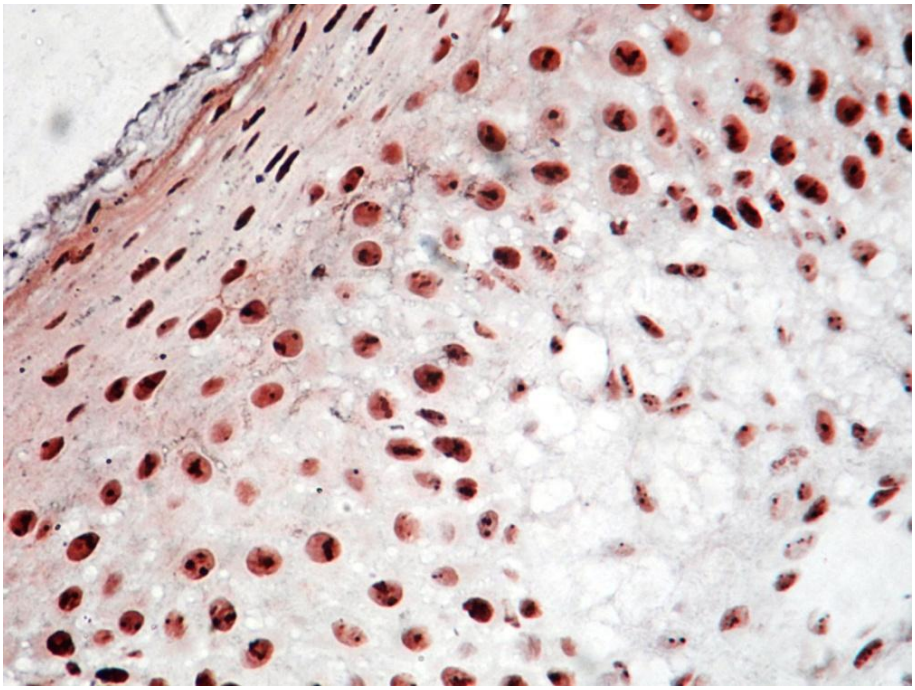


Fig 7. AgNORs in normal oral mucosa (x 400)



RESULTS

In the present study, a total of 94 cases of histopathologically proven OSCC were included, among which 30 patients had OSCC of buccal mucosa (Group I), 24 patients had OSCC of alveolar mucosa (Group II), 25 patients had OSCC of tongue, 15 patients had OSCC of retromolar trigone area (Group IV) (***table 1,2,3,4***). Each group was further divided into sub-groups based on etiological factors. Sub-group IA included 8 cases of OSCC of buccal mucosa with habit of betel quid chewing, Sub-group IB included 15 cases with etiology of mawa/gutka chewing, Sub-group IC included 7 cases with etiology of smoking ***(table 1)***. Group II had etiological factors similar to group I and was similarly subdivided into Sub-group IIA, IIB, IIC with 13, 6, 5 cases in each Sub-group respectively ***(table 2)***. Group III did not have cases with etiology of betel quid chewing but included cases with etiology of trauma. It was hence sub-divided into Sub-group IIIA with etiology of mawa/gutka chewing, Sub-group IIIB with etiology of trauma and Sub-group IIIC with etiology of smoking (***table 3).*** Group IV was subdivided similar to group I and II (***table 4).***Table 1, 2, 3, 4 represents the master chart for Group I, II, III, IV respectively.

The histological sections from blocks retrieved from department archives for all these cases were stained for visualizing AgNORS and the mean AgNOR count was calculated for each caase and compared.

The average mean AgNOR counts for Group I, Group II, Group III and Group IV were found to be 3.1883, 3.1350, 3.6724 and 3.1507 respectively. The mean AgNOR count for Group IV was significantly higher than Group I, II and III with P value = 0.001 ($P<0.05$). ***(table 9,10,11 and Fig 9)***

In Group I, the average of mean AgNOR counts for each Sub-groups revealed a significantly higher value for Sub-group IC (mAgNOR=3.604) when compared to Sub-group IA (mAgNOR=2.9975) and Sub-group IB (mAgNOR=3.096) with P value less than 0.05.***(table 12,13 and Fig 10)***

In Group II, there was no significant difference in the mean AgNOR counts among Sub-Group IIA, IIB, IIC with P value greater than 0.05.***(table 14 and Fig 11)***

In Group III, Sub-group IIIB (mAgNOR=4.4138) showed significantly higher mean AgNOR counts when compared to Sub-group IIIA (mAgNOR=3.1375) and Sub-group IIIC (mAgNOR=3.2100) with P value 0.000. (P<0.05)***(table 15,16 and Fig 12)***

In Group IV, there was no significant difference in the mean AgNOR counts between the Sub-groups IVA, IVB, IVC and IVD. (P>0.05) ***(table 17 and Fig 13)***

When comparing the mean AgNOR counts between the Groups for etiology of betel quid chewing, i.e., Sub-group IA, IIA and IVA, there was no statistically significant difference***(table 18).***Similarly, when comparing the mean AgNOR counts between the groups for etiology of mawa/gutka chewing, i.e., Sub-group IB, IIA,IIIB, there was no statistically significant difference.(***table 19)***

Table 5 : mAgNOR COUNTS IN GROUP I – Carcinoma of BUCCAL MUCOSA

SNo	HP No.	ETIOLOGY	mAgNOR count
1.	14150	Betel quid chewing	2.55
2.	14312	Betel quid chewing	3.87
3.	14318	Betel quid chewing	3.64
4.	14327	Betel quid chewing	2.55
5.	14350	Betel quid chewing	2.84
6.	14473	Betel quid chewing	2.92
7.	14501	Betel quid chewing	2.67
8.	14700	Betel quid chewing	2.94
9.	14248	Maawa/gutka chewing	3.19
10.	14250	Maawa/gutka chewing	2.42
11.	14257	Maawa/gutka chewing	2.76
12.	14302	Maawa/gutka chewing	3.39
13.	14336	Maawa/gutka chewing	2.98
14.	14399	Maawa/gutka chewing	2.83
15.	14556	Maawa/gutka chewing	3.64
16.	14633	Maawa/gutka chewing	2.96
17.	14639	Maawa/gutka chewing	3.01
18.	14662	Maawa/gutka chewing	3.28
19.	14680	Maawa/gutka chewing	2.80
20.	14687	Maawa/gutka chewing	3.28
21.	14697	Maawa/gutka chewing	3.32

22.	14708	Maawa/gutka chewing	2.26
23.	14711	Maawa/gutka chewing	4.32
24.	14551	Smoking	3.64
25.	14615	Smoking	3.65
26.	14641	Smoking	3.63
27.	14676	Smoking	3.68
28.	14677	Smoking	3.66
29.	14690	Smoking	3.32
30.	14727	Smoking	3.65

SUB- GROUP I A : Etiology of betel quid chewing

SUB-GROUP I B : Etiology of maawa or gutka chewing

SUB-GROUP I C : Etiology of smoking

Table 6: mAgNOR COUNTS IN GROUP II – Carcinoma of ALVEOLAR MUCOSA

SNo	HP No	ETIOLOGY	mAgNOR count
1.	14300	Betel quid chewing	3.48
2.	14329	Betel quid chewing	2.97
3.	14371	Betel quid chewing	2.64
4.	14439	Betel quid chewing	3.44
5.	14444	Betel quid chewing	2.80
6.	14458	Betel quid chewing	3.14
7.	14470	Betel quid chewing	3.58

8.	14472	Betel quid chewing	2.41
9.	14507	Betel quid chewing	2.22
10.	14522	Betel quid chewing	3.48
11.	14572	Betel quid chewing	2.70
12.	14630	Betel quid chewing	2.65
13.	14723	Betel quid chewing	2.92
14.	14384	Maawa/gutka chewing	3.34
15.	14560	Maawa/gutka chewing	3.75
16.	14561	Maawa/gutka chewing	3.12
17.	14591	Maawa/gutka chewing	3.33
18.	14628	Maawa/gutka chewing	3.43
19.	14675	Maawa/gutka chewing	3.30
20.	14383	Smoking	3.59
21.	14440	Smoking	3.64
22.	14559	Smoking	3.28
23.	14597	Smoking	3.28
24.	14603	Smoking	2.75

SUB-GROUP IIA : Etiology of Betel quid chewing habit

SUB-GROUP IIB : Etiology of maawa/gutka chewing habit

SUB-GROUP IIC : Etiology of smoking

Table 7: mAgNOR COUNTS IN GROUP III – Carcinoma of TONGUE

SNo	HP No	ETIOLOGY	mAgNOR count
1.	14265	Maawa/gutka chewing	2.58
2.	14359	Maawa/gutka chewing	3.86
3.	14421	Maawa/gutka chewing	3.22
4.	14477	Maawa/gutka chewing	3.30
5.	14645	Maawa/gutka chewing	3.32
6.	14671	Maawa/gutka chewing	2.80
7.	14692	Maawa/gutka chewing	3.28
8.	14726	Maawa/gutka chewing	2.74
9.	14151	Trauma	4.34
10.	14347	Trauma	4.03
11.	14386	Trauma	4.28
12.	14468	Trauma	3.88
13.	14521	Trauma	4.46
14.	14528	Trauma	4.76
15.	14598	Trauma	3.88
16.	14606	Trauma	4.28
17.	14622	Trauma	3.98
18.	14661	Trauma	4.44
19.	14669	Trauma	3.96
20.	14702	Trauma	3.16
21.	14736	Trauma	4.42
22.	14398	Smoking	3.08

23.	14410	Smoking	3.52
24.	14704	Smoking	3.06
25.	14707	Smoking	3.18

SUB-GROUP IIIA : Etiology of maawa/gutka chewing

SUB-GROUP IIIB : Etiology of trauma

SUB-GROUP IIIC : Etiology of smoking

Table 8: mAgNOR COUNTS IN GROUP IV – Carcinoma of RETROMOLAR TRIGONE AREA

SNo	HP No	ETIOLOGY	mAgNOR
1.	14375	Betel quid chewing	3.44
2.	14497	Betel quid chewing	3.44
3.	14523	Betel quid chewing	2.70
4.	14636	Betel quid chewing	2.23
5.	14667	Betel quid chewing	2.63
6.	14280	Maawa/gutka chewing	3.14
7.	14288	Maawa/gutka chewing	3.46
8.	14328	Maawa/gutka chewing	3.58
9.	14395	Maawa/gutka chewing	3.14
10.	14460	Maawa/gutka chewing	3.52
11.	14461	Maawa/gutka chewing	3.56

12.	14563	Maawa/gutka chewing	3.88
13.	14706	Maawa/gutka chewing	3.10
14.	14441	Smoking	2.68
15.	14446	Smoking	2.76

SUB-GROUP IVA : Etiology of Betel quid chewing

SUB-GROUP IVB : Etiology of Maawa/gutka chewing

SUB-GROUP IVC : Etiology of smokin

Table 9:STATISTICAL ANALYSIS AND RESULTS;

Descriptive Statistics of the variables with Minimum,Maximum,Mean and Standard deviation					
	Number	Minimum	Maximum	Mean mAgNOR count	Standard Deviation
GROUP I	30	2.26	4.32	3.1883	.48834
GROUP II	24	2.22	3.75	3.1350	.41558
GROUP III	25	2.58	4.76	3.6724	.62120
GROUP IV	15	2.23	3.88	3.1507	.46153
SGIA	8	2.55	3.87	2.9975	.49511
SGIB	15	2.26	4.32	3.0960	.49812
SGIC	7	3.32	3.68	3.6043	.12634
SGIIA	13	2.22	3.58	2.9562	.44077
SGIIB	6	3.12	3.75	3.3783	.20856
SGIIC	5	2.75	3.64	3.3080	.35450
SGIIIA	8	2.58	3.86	3.1375	.41258
SGIIIB	13	3.16	4.76	4.1438	.39790
SGIIIC	4	3.06	3.52	3.2100	.21323
SGIVA	5	2.23	3.44	2.8880	.53486
SGIVB	8	3.10	3.88	3.4225	.27453
SGIVC	2	2.68	2.76	2.7200	.05657

SG – Sub-Group

Table 10: COMPARISON OF MEAN AgNOR COUNT BETWEEN THE STUDY GROUPS

The mean value of AgNOR count for four groups were compared by one way analysis of variance (ANOVA)

ANOVA between the groups				
	Mean	Standard Deviation	F value	P value
GROUP I	3.1883	.48834	6.262	.001
GROUP II	3.1350	.41558		
GROUP III	3.6724	.62120		
GROUP IV	3.1507	.46153		

INFERENCE :

The results indicate P value equal to 0.001. There is significant difference between groups ($P<0.05$) with respect to mean AgNOR counts.

Table 11: MULTIPLE COMPARISON OF MEAN AgNOR COUNTS BETWEEN STUDY GROUPS

Multiple comparisons are made between different study groups for significant difference in the mean AgNOR counts.

Multiple Comparisons						
GROUP I, GROUP II, GROUP III, GROUP IV Tukey HSD PROCEDURE						
(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Standard Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
GROUP I	GROUP II	.05333	.13884	.981	-.3101	.4168
	GROUP III	-.48407	.13729	.004	-.8434	-.1247
	GROUP IV	.03767	.16032	.995	-.3820	.4573
GROUP II	GROUP I	-.05333	.13884	.981	-.4168	.3101
	GROUP III	-.53740	.14488	.002	-.9166	-.1582
	GROUP IV	-.01567	.16686	1.000	-.4525	.4211
GROUP III	**GROUP I**	.48407	.13729	**.004**	.1247	.8434
	GROUP II	.53740	.14488	**.002**	.1582	.9166
	GROUP IV	.52173	.16558	**.012**	.0883	.9552
GROUP IV	GROUP I	-.03767	.16032	.995	-.4573	.3820
	GROUP II	.01567	.16686	1.000	-.4211	.4525
	GROUP III	-.52173	.16558	.012	-.9552	-.0883

INFERENCE:

The mean AgNOR counts of group III is significantly different from group I, II and III ($P<0.05$). Hence, the mean AgNOR counts for squamous cell carcinoma of tongue is significantly higher than the mean AgNOR counts in squamous cell carcinoma of other sites.

COMPARISON OF MEAN AgNOR COUNT BETWEEN THE SUB-GROUPS IN GROUP I

Table 12: ANOVA between the Sub- groups of GROUP 1				
	Mean	Standard Deviation	F value	P value
SG IA	2.9975	.49511	4.164	.027
SG IB	3.0960	.49812		
SG IC	3.6043	.12634		

SG – Sub-group

Table 13: Multiple Comparisons between sub-groups of group I						
Turkey HSD procedure						
(I) SG I ABC	(J) SG I ABC	Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
SG IA	SG IB	-.09850	.19370	.868	-.5788	.3818
	SG IC	-.60679	.22899	.034	-1.1745	-.0390
SG IB	SG IA	.09850	.19370	.868	-.3818	.5788
	SG IC	-.50829	.20252	.047	-1.0104	-.0061
SG IC	SG IA	.60679	.22899	.034	.0390	1.1745
	SG IB	.50829	.20252	.047	.0061	1.0104

SG – Sub-group

INFERENCE:

The mean AgNOR counts for different sub-groups within Group I are compared using ANOVA and the P value of 0.027 denotes that the difference is statistically significant ($P<0.05$)

Multiple comparisons within sub-groups of group I by Turkey HSD showed significant difference in mean AgNOR counts between sub groups IA & IC , IB & IC ($P<0.05$) and difference between IA & IB is not significant.

The mean AgNOR counts for carcinoma of buccal mucosa with etiology of smoking significantly higher than those with etiology of betel quid chewing and maawa/gutka chewing.

Table 14: COMPARISON OF MEAN AgNOR COUNT BETWEEN THE SUB-GROUPS IN GROUP II

ANOVA between the Sub- groups in Group II				
	Mean	Std. Deviation	F valve	P value
SG IIA	2.9562	.44077	3.168	0.06*
SG IIB	3.3783	.20856		
SG IIC	3.3080	.35450		
* Not Significant				

SG – Sub Group

INFERENCE:

There is no significant difference in the mean AgNOR values between different sub-groups in group II. ($P>0.05$)

There is no significant difference in mean AgNOR counts in Carcinoma of alveolar mucosa due to different etiologies.

COMPARISON OF MEAN AgNOR COUNT BETWEEN THE SUB-GROUPS IN GROUP III

Table 15 :ANOVA between the Sub- groups in Group III

	Mean	Standard Deviation	F value	P value
SG IIIA	3.1375	.41258	20.561	.000
SG IIIB	4.1438	.39790		
SG IIIC	3.2100	.21323		

SG- Sub Group

Table 16: Multiple Comparisons between Sub-groups in Group III

Group III
Tukey HSD

(I) SG III ABC	(J) SG III ABC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
SG IIIA	SG IIIB	-1.00635	.17212	.000	-1.4387	-.5740
	SG IIIC	-.07250	.23456	.949	-.6617	.5167
SG IIIB	SG IIIA	1.00635	.17212	.000	.5740	1.4387
	SG IIIC	.93385	.21901	.001	.3837	1.4840
SG IIIC	SG IIIA	.07250	.23456	.949	-.5167	.6617
	SG IIIB	-.93385	.21901	.001	-1.4840	-.3837

SG- Sub Group

INFERENCE :

The mean AgNOR counts for different sub-groups within Group III are compared using ANOVA and the P value of 0.000 denotes that the difference is statistically significant ($P<0.05$)

Multiple comparison between the groups using Turkey HSD procedure shows the difference in mean AgNOR count between 3A & 3C is not significant but IIIA & IIIB ($P = 0.000$), IIIB & IIIC ($P= 0.001$) are significantly different ($P<0.05$).

In Carcinoma of tongue, cases with trauma as the etiological factor show significantly higher mean AgNOR counts when compared to cases with smoking or betel quid chewing habit.

Table 17:COMPARISON OF MEAN AgNOR COUNT BETWEEN THE SUB-GROUPS IN GROUP IV

ANOVA between the Sub- groups in Group IV				
	Mean	Std. Deviation	F value	Sig.
SG IVA	2.8880	.53486	4.682	.066
SG IVB	3.4225	.27453		
SG IVC	2.7200	.05657		

SG – Sub Group

INFERENCE:

There is no significant difference in the mean AgNOR values between different sub-groups in group IV. (P value >0.05)

There is no significant difference in mean AgNOR counts in Carcinoma of retromolar trigone region due to different etiologies.

Table 18: Comparison of mean AgNOR counts between groups in patients with Betel quid chewing habit

ANOVA between the Sub- groups- IA IIA IVA				
	Mean	Std. Deviation	F	Sig.
SG IA	2.9975	.49511	.082	0.921*
SG IIA	2.9562	.44077		
SG IVA	2.8880	.53486		
* Not Significant				

INFERENCE :

There is no significant difference in the mean AgNOR counts between the sub-groups IA, IIA, IVA. (P value >0.05)

There is no significant difference in the cell proliferation rate in oral squamous cell carcinoma of buccal mucosa, alveolar mucosa and retromolar area among betelquid chewers.

Table 19: Comparison of mean AgNOR counts between groups in patients with maawa/gutka habit

ANOVA between the Sub- groups- IB IIB IIIA IVB				
	Mean	Std. Deviation	F	Sig.
SG IB	3.0960	.49812	1.552	.219
SG IIB	3.3783	.20856		
SG IIIA	3.1375	.41258		
SG IVB	3.4225	.27453		
* Not Significant				

INFERENCE:

There is no significant difference in the mean AgNOR counts between the sub-groups IIA, IIB, IIIA and IVB. (P value >0.05)

There is no significant difference in the cell proliferation rate in oral squamous cell carcinoma of buccal mucosa, alveolar mucosa, tongue and retromolar area among maawa or gutka chewers.

GRAPHICAL REPRESENTATION OF RESULTS

Fig 9. Comparison of mAgNOR counts between the four groups (P<0.05)

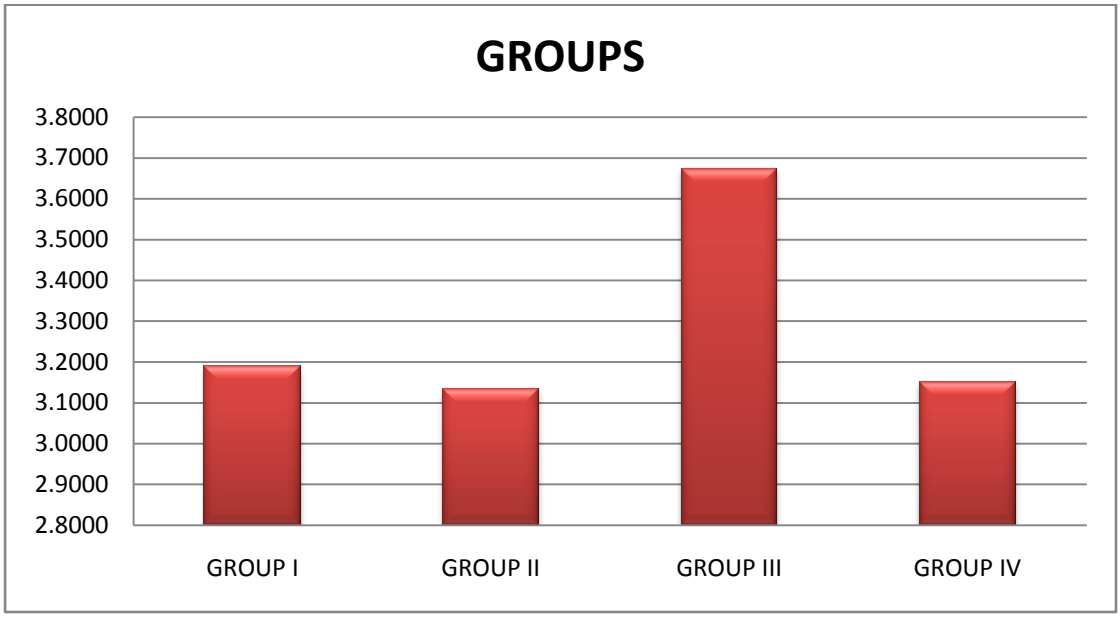


Fig 10. Comparison of mAgNOR counts in Group I sub-groups (P<0.05)

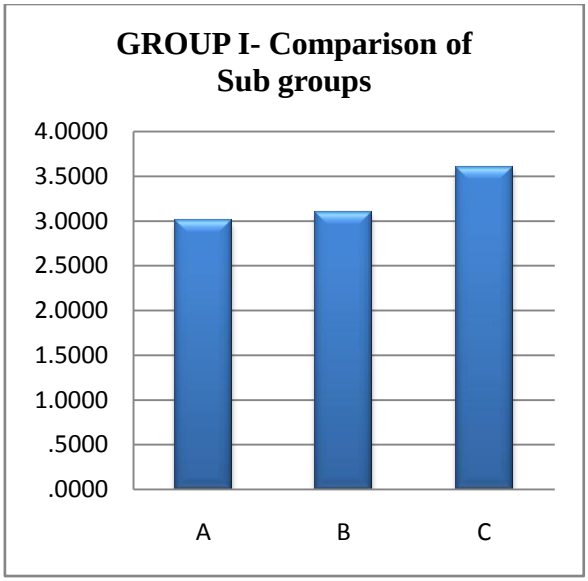


Fig 11. Comparison of mAgNOR counts in Group II sub-groups (P>0.05) (not significant)

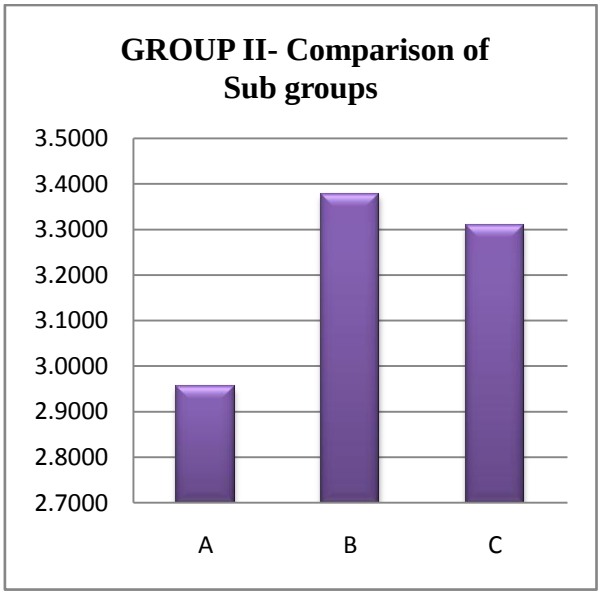


Fig 12. Comparison of mAgNOR counts in Group III sub-groups (P<0.05)

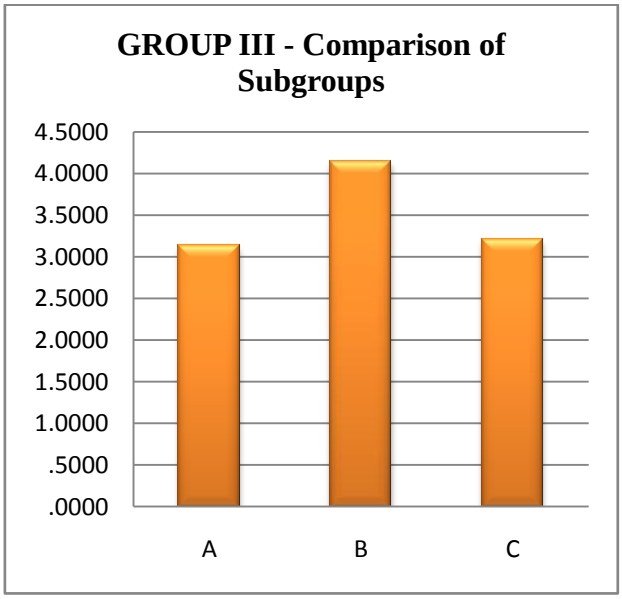
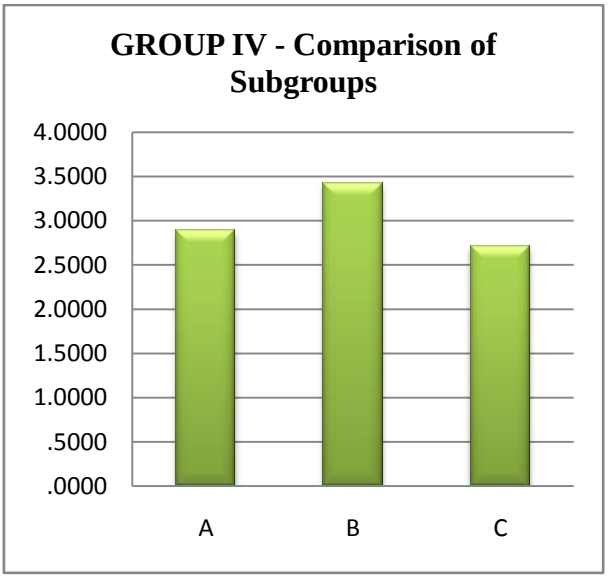


Fig 13. Comparison of mAgNOR couns in Group IV sub-groups (P>0.05) (not significant)



DISCUSSION

Oral squamous cell carcinoma (OSCC) is associated with increasing mortality rates in India.[2] The prognosis of oral cancer depends on many clinicopathological parameters including site of origin and etiolological factors which has been supported by studies by ***Shiu S et al,***[48] ***2004, Garzino-Demo P,***[17] ***et al 2006, Subapriya R et al,***[49] ***2007***, etc,. Cell proliferation rate has been considered as one of the determinants of prognosis in oral cancer**.**[24] In this study, we have evaluated the cell proliferation rate in some of the common subsites of OSCC and also studied the influence of some of the etiological factors on cell proliferation rate in different subsites of OSCC and therefore indirectly determining the prognostic implication of variation in subsites and etiologies of oral cancer.

There are many methods to determine the cell proliferation rate.[4,25] However in this study we used quantification of silver-stained nucleolar organizer regions, as it is cheaper, reliable, less time consuming method and also the results obtained are comparable to other proliferative markers which are determined by complex procedures.[12] The expression of AgNOR protein is especially associated with rapidity of cell proliferation and a higher AgNOR count is associated with aggressive phenotype. There are many studies involving AgNORs as cell proliferation marker in oral cancer. [11,32,37,38] A standardized method for AgNOR quantification is available and it produced reproducible results.[30]

In this present study we included 94 cases of oral squamous cell carcinoma involving buccal mucosa, alveolus, tongue and retromolar trigone area. The commonest site invoved was buccal mucosa followed by almost equal number of cases with carcinoma of tongue and alveolus. The predominance in the involvement

of buccal mucosa is in accordance with studies conducted by ***Kaur J et al,[40] 1994, Manu V et al,[50] 2006 .***

According to studies conducted by ***Garzino-Demo P et al,[17] 2006, Rusthoven K et al,[18] 2008*** there is difference in prognosis for different subsites of oral carcinoma. ***Ratuva et al,[51] 2007*** have attributed the difference in prognosis to the type of oral epithelium – keratinized, non keratinized and specialized mucosa, as these were found to have different turnover rates and the keratin layer acts as a barrier to outside environment. None of these studies compared the cell proliferation rate as a cause for difference in prognosis for these sub-sites. However, in this study we found a significant difference in cell proliferation rate for carcinoma of buccal mucosa, alveolus, tongue and retromolar trigone area as shown by the significant difference in the mean AgNOR counts for these subsites.

Carcinoma of tongue showed significantly higher mean AgNOR counts when compared to other subsites. This correlates with the poor prognosis of tongue cancer as established by studies by ***Garzino-Demo P et al,[17] in 2006, Rusthoven K et al,[18] in 2008***. Further studies are required comparing the cell proliferation in different subsites and to establish difference in cell proliferation rate as a cause for difference in prognosis among different subsites.

Various etilogical factors have been considered to influence the prognosis of oral squamous cell carcinoma. Various studies included betel quid chewing, mawa/gutka chewing, snuff dipping, smoking and alcohol as the chief etiological factors.[40-47]The previous demographic data collected from patients in our department suggested that cases of oral squamous cell carcinoma reporting to our department had the habits of betel quid chewing with tobacco, maawa or gutka chewing, smoking and

use of oral snuff. Since there were very few cases with etiology of using oral snuff during the period of our study, comparable results couldn't be obtained and hence eliminated from our study. Although alcohol is considered as the etiological factor in many of the studies in literature, use of alcohol alone does not cause cell proliferation per se and it acts synergistically when combined with the habit of smoking or chewing.[41,44] Alcohol acts as a solvent for many carcinogens including tobacco carcinogens, thereby enhancing tissue penetration.[50] During our study period none of the patients reported had OSCC and habit of alcoholism alone. The patients who had chronic alcoholism along with other etilological factors included in our study were eliminated.

Another common etiological factor that we come across in cases reporting to our department with squamous cell carcinoma of tongue is trauma. Although it is said that chronic irritation to oral mucosa by the contents of quid is a source of trauma and causes oral cancer, there are a few number of studies, as revealed by pubmed search, considering trauma as one of the etiological factor for oral cancer.[52,53,54] In our study, mean AgNOR counts in squamous cell carcinoma of tongue due to trauma was significantly higher as compared to other etiological factors. Hence trauma should be considered in studies conducted on tongue cancer as it may have a prognostic implication.

In this study, there was no significant difference in mean AgNOR counts due to etiologies in other subsites except in carcinoma of buccal mucosa where smoking showed greater cell proliferation index when compared to other etiologies. But studies conducted by ***Znaor A et al,[42] 2003 and Lin WJ et al,[44] 2011*** showed betel quid chewing to have highest risk when compared to smoking in oral cavity, but cell proliferation or prognosis was not considered in either of the studies.

Thus, among the four subsites considered in this study, squamous cell carcinoma of tongue showed significantly higher cell proliferation rate which explains its poor prognosis and trauma is one of the often neglected but chief etiological factor for tongue cancer as it causes greater cell proliferation when compared to other etiologies. Further studies are implicated to associate higher cell proliferation rate to poor prognosis in tongue squamous cell carcinoma and also considering trauma as one of the chief etiological factor.

SUMMARY AND CONCLUSION

AgNORs are considered to be important cell proliferation marker and a predictor of prognosis in OSCC. The higher the cell proliferation rate, higher is the mean AgNOR count and poorer is the prognosis.

In the present study, we have included 94 patients with OSCC and compared the cell proliferation rate by mean AgNOR counts in some of the common subsites of OSCC and also studied the influence of the etiological factors on cell proliferation rate which in turn reflects the prognosis in different subsites of OSCC. Of the four subsites included OSCC of tongue showed significantly higher mean AgNOR counts when compared to OSCC of buccal mucosa, alveolar mucosa and retromolar trigone area ($P<0.05$) and in OSCC of tongue, trauma showed significantly greater mean AgNOR counts when compared to other etiological factors. Similarly in OSCC of buccal mucosa smoking showed greater mean AgNOR counts when compared to other etiological factors ($P<0.05$).

From the results of this study the following conclusion can be drawn:

1. OSCC of tongue shows greater cell proliferation rate and thus correlating with its poorer prognosis as established by many other studies.

2. Trauma causes significantly higher cell proliferation in OSCC of tongue and hence should be considered as one of the important prognostic determinant in further studies involving OSCC of tongue.

Further studies are required implicating the difference in cell proliferation rate due to different etiological factors in OSCC sub-sites to establish conclusive results.

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Appendix 1

INFORMATION SHEET

- We are conducting a study on **"CELL PROLIFERATION IN ORAL SQUAMOUS CELL CARCINOMA IN DIFFERENT SUBSITES**". For that study, we are selecting patients.
- The purpose of this study is to compare the cell proliferative index and to evaluate the role of causative factors among different sub sites within the oral cavity.
- The identity of the patients participating in the research will be kept confidential throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.
- Taking part in the study is voluntary. You are free to decide whether to participate in the study or to withdraw at any time; your decision will not result in any loss of benefits to which you are otherwise entitled.
- The results of the special study may be intimated to you at the end of the study period or during the study if anything is found abnormal which may aid in the management or treatment.

Name of the patient Signature / Thumb impression

Name of the investigator Signature Date

Appendix 2
INFORMED CONSENT FORM

STUDY TITLE:

"CELL PROLIFERATION IN ORAL SQUAMOUS CELL CARCINOMA IN DIFFERENT SUBSITES",

Name: O.P.No: H/P no:

Address: S. No: Group no:

Age / Sex:

Tel. no:

I, _____ age _____ years exercising my free power of choice, hereby give my consent to be included as a participant in the study **"CELL PROLIFERATION IN ORAL SQUAMOUS CELL CARCINOMA IN DIFFERENT SUBSITES**"

I agree to the following:

- I have been informed to my satisfaction about the purpose of the study and study procedures including investigations to monitor and safeguard my body function.
- I have been informed that the biopsy tissue specimen taken for diagnosis purpose will only be used for the study and no other extra tissue specimen will be taken from me for the purpose of study.
- I agree to share details of my personal habits for the purpose of the study and I am told that the investigating doctor and institution will keep my identity confidential.
- I hereby give permission to use my medical records for research purpose. I am told that the medical details will also be kept confidential.

Name of the patient Signature / Thumb impression

Name of the investigator Signature Date

ஒப்புதல் படிவம்

ஆராய்ச்சியின் தலைப்பு

வாயில் வரும் வாய் இறுக்குநோய், வாய்புற்றுநோய் மற்றும் நோயற்ற நபர்களின் குருதி ஊநீரில் உள்ள பீட்டா – கரோட்டினின் அளவை அறியும் ஆராய்ச்சி

பெயர் : புற நோயாளி எண்:

முகவரி : எண் :

வயது/ பால்:

தொலைபேசி எண் :

நான் ..... வயது ..... வருடம்

எனது முழுமனதுடன் வாயில் வரும் ''வாய் இறுக்குநோய், வாய்புற்றுநோய் மற்றும் நோயற்ற நபர்களின் குருதி ஊநீரில் உள்ள பீட்டா – கரோட்டினின் அளவை அறியும் ஆராய்ச்சி''யில் பங்கு பெற சம்மதிக்கிறேன்.

நான் கீழ்கண்டவற்றுக்கு சம்மதிக்கிறேன்

- நான் இந்த ஆராய்ச்சியின் நோக்கம் மற்றும் ஆராய்ச்சியின் முறைகள் பற்றி முழுமையாக தெரிவிக்கப்பட்டுள்ளேன்.
- நான் என் குருதி ஊநீரை ஆராய்ச்சிக்குப் பயன்படுத்த ஒப்புதல் அளிக்கிறேன்.
- நான் என்னுடைய மருத்துவருக்கு முழு ஒத்துழைப்பு தர சம்மதிக்கிறேன். நான் ஏதேனும் உடல் அசௌகரியம் ஏற்பட்டால் மருத்துவரிடம் உடனடியாகத் தெரிவிப்பேன்.
- நான் மருத்துவரிடம் இதற்கு முன்னர் மற்றும் தற்போது உட்கொள்ளும் மருந்துகள் பற்றியும், எனக்கு உள்ள மற்ற வியாதிகள் பற்றியும் முழுமையாகத் தெரிவிப்பேன்
- நான் ஆராய்ச்சியின் தன்மைக்கேற்ப தேவைப்படும் நேரத்தில் மருத்துவரைத் தொடர்புக் கொள்வேன்.
- நான் என் மருத்துவப் படிவங்களை ஆராய்ச்சிக்காக உபயோகப் படுத்த அனுமதிக்கிறேன். நான் மருத்துவர் மற்றும் மருத்துவமனை என்னுடைய அடையாளத்தை ரகசியமாக வைப்பார்கள் என்று கூறப்பட்டுள்ளேன்.

.....	.....	.....
நோயாளியின் பெயர்	கையொப்பம்/கைரேகை	தேதி
.....	.....	.....
ஆராய்ச்சியாளரின் பெயர்	கையொப்பம்	தேதி

ஆராய்ச்சி பற்றிய தகவல் படிவம்

1. வாயில் வரும் ‘‘வாய் இறுக்குநோய், வாய்புற்றுநோய் மற்றும் நோயற்ற நபர்களின் குருதி ஊநீரில் உள்ள பீட்டா – கரோட்டினின்’’ அளவை அறிதல் குறித்து ஆராய்ச்சி செய்யும் பொருட்டு தமிழ்நாடு அரசு பல் மருத்துவமனை மற்றும் கல்லூரிக்கு வரும் நோயாளிகள் தேர்வு செய்யப்படுகிறார்கள்.

2. இந்த ஆராய்ச்சியின் நோக்கம் வாயில் வரும் ‘‘வாய் இறுக்குநோய், வாய்புற்றுநோய் மற்றும் நோயற்ற நபர்களின் குருதி ஊநீரில் உள்ள பீட்டா – கரோட்டினின்’’ அளவை அறிதல் ஆகும்.

3. நோயாளி பற்றிய குறிப்புகள் பிறர் அறியாவண்ணம் ஆராய்ச்சி முடியும்வரை இரகசியமாக பாதுகாக்கப்படும். அதை வெளியிடும் நேரத்தில் எந்த நோயாளியின் தனி அடையாளங்களும் வெளியிட வாய்ப்பு கிடையாது.

4. இந்த ஆராய்ச்சியில் இங்கு பெறுவது நோயாளியின் தனிப்பட்ட முடிவு மற்றும் நோயாளிகள் இந்த ஆராய்ச்சியில் இருந்து எப்பொழுது வேண்டுமானாலும் விலகிக் கொள்ளலாம். நோயாளியின் இந்த தீடீர் முடிவு, அவருக்கோ அல்லது ஆராய்ச்சியாளருக்கோ எந்தவித பாதிப்பும் ஏற்படுத்தாது என்பதை தெரியப் படுத்துகிறோம்.

5. இந்த ஆராய்ச்சியின் முடிவுகள் நோயாளிகளுக்கு ஆராய்ச்சி முடியும் தறுவாயிலோ அல்லது இடையிலோ தெரிவிக்கப்படும். ஆராய்ச்சியின்பொழுது ஏதும் பின் விளைவுகள் ஏற்பட்டால் அதை சரி செய்ய தகுந்த உதவிகள் அல்லது தேவையான சிகிச்சைகள் உடனடியாக மேற்கொள்ளப்படும்.

நோயாளியின் பெயர்: **கையொப்பம்/கைரேகை**

Appendix 3 : To determine mean AgNOR counts in 100 cells

S.NO :

GROUP :

HP NO :

ETIOLOGY :

mAgNOR count :

APPENDIX IV – COMMENTS AND SUGGESTIONS BY SUPERVISOR

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Comment 1.	1. Principal should be acknowledged first, followed by dissertation screening committee and ethical committee, and then proceed with acknowledging the rest. 2. Remove the word 'guide' where ever necessary and replace with just 'supervisor' 3. I thank Dr. I. Ponniah for his help in the dissertation and overall guidance during my MDS course. 4. I am not the principal investigator. 5. In the declaration by the student, state firmly that I (Dr. Arya.A.N) is entirely responsible for any ethical violations (if any) and it does not have any binding on my supervisor.	Corrections made as suggested Changed 'guide' to 'supervisor' Corrections made Principal investigator changed to co-investigator Separate declaration by candidate enclosed accordingly
Comment 2a	In the abstract section, include aim and objective(s).	Objective added

SUGGESTIONS FOR OTHER SECTIONS - NIL

APPENDIX - V

HISTORY AND PRE-HISTORY OF DISSERTATION

Whether a course in research methodology was attended?	Yes, from 31-01-2012 to 04-02-2012
Whether adequate training obtained before starting dissertation with regard to conception, design and literature search?	Yes, it was given in the first year of MDS course.
When was the dissertation topic selected?	13-11-2012
Whether topic selection was discussed with the guide?	Yes
Whether a research question put forth?	Yes
Whether aim and objective was discussed and got approved from the guide?	Yes, 25-11-2012
Whether materials and methods discussed and got approved?	Yes, 05-12-2012
Whether the topic got approved from the dissertation screening and ethical committees?	Yes, 25-01-2012
Whether the study protocol differed from that initially conceived and approved by the ethical committee? If yes, state the reasons.	Number of cases were increased as there weren't adequate number of cases for comparison in certain sub groups
Whether the data for review of literature discussed with the guide?	No.
Whether literature review was shown to the guide, and if yes, when it was shown?	YES on 26-12-2012
Who did data analysis and interpretation?	I did data analysis and interpretation.
Who did statistical analysis and when it was completed?	STATISTICIAN on 5-12-2012
Whether bibliography discussed with the guide?	No.
Whether printed copy of bibliographies provided to the guide?	Yes
When was the following textual content shown to the guide? When was returned to you after correction? Whether the following were approved on initial submission or required number of corrections?	
(i) Introduction	27-08-2012 (2 corrections)
(ii) Aim and Objective	25-11-2012 (2 corrections)
(iii) Review of Literature	26-12-2012
(iv) Material and Methods	05-12-2012 (2 corrections)
(v) Results	26-12-2012
(vi) Discussion	26-12-2012
(vii) Conclusion	26-12-2012
(viii) Bibliography	26-12-2012
Whether the above sections were edited for language and intellectual content? Whether the final document was checked for overlap with previous work by others?	Only for introduction No
When was it shown and got approved?	shown on 26-12-2012 at 11.30 am and got approved at 11.50 am
Whether answer was found to the research question?	YES