

To assess the utility of actual proliferation index measured by expression of ‘Agnor and Ki-67’ in histopathologically negative surgical margins of oral squamous cell carcinoma as a modality for prediction of recurrence.

Abstract:

Background: Oral squamous cell carcinoma is most common malignancy of head and neck which is the major cause of death. Recurrence is the major important prognostic factor in the survival of patients and tumor stage, degree of differentiation of neoplastic cells, pN stage resected margins, and lymphovascular invasion are the factors for recurrence. The rate of recurrence varies from 18 to 76% for patients who underwent standard treatment. Histological assessment of surgical margins has a major role to predict the recurrence. But in spite of negative histological surgical margins recurrence rate is high, as there are genetic alterations in these margins which are not detected by routine histopathology. Even when surgical margins are free histopathologically the local recurrence rate is 10 to 30 percent.

That's why molecular evaluation of these margins should be done. Many immunohistochemical studies are done to assess surgical margins of OSCC but no study is carried out to assess the proliferative index in surgical margins. Therefore this study will help to improve management and predict the recurrence of OSCC.

Objectives: In this study we aim to assess the utility of “actual proliferation index” measured by expression of “Agnor” and “Ki-67” in histopathologically negative “surgical margins” of “oral squamous cell carcinoma” as a modality for prediction of recurrence.

Methodology: The resected surgical margins of the oral squamous cell carcinoma cases which are operated in Sharad Pawar dental college will be selected as samples. The anterior, posterior, inferior and superior margin of resected specimens will be stained by H&E staining. Immunohistochemical staining by ki-67 antibody will be done in the negative histopathological surgical margins. The same sections will be stained with AgNOR staining. Both sections will be evaluated. Actual proliferative index will be calculated with the given formula

Ki67 Labeling index:

$$\text{Ki67 LI} = \frac{\text{“Number of Ki67 positive cells”} \times 100}{\text{Total number of tumor cells observed}}$$

“Actual Proliferative Index (PI)” = “Ki-67” or MIB-1 LI \times AgNOR count ’

Recurrence of tumor will be observed during first two years period after surgical procedure.

Expected Results: The “actual proliferative index” calculated by “Ki-67 labelling index” and “AgNOR count expression in histopathologically” negative surgical margins of OSCC will be more in recurrence patients as compared to patients who do not have recurrence.

Conclusion: The API values measured by assessing the Agnor and Ki67 expression, in histopathological negative surgical margins of OSCC will be compared between with values of patient with recurrence.

Keywords: “Oral squamous cell carcinoma”, Negative surgical margins, Recurrence.

Introduction:

“Oral squamous cell carcinoma” is the major malignancy reported in “South east Asian population”. The treatment of choice is radical neck dissection. In spite of advances in treatment modalities 5 year survival rate has not improved significantly. This is because of local recurrence. It is observed that 50 percent of locally recurrent tumors arise in surgical margins that are not detected by routine histological examination . (1). If the primary tumor is not completely resected there are more chances of recurrence and thereby reducing survival rate.[1,2] It is very important clinically to attain negative histological margins with the help of routine hematoxylin and Eosin staining as well as with molecular markers.

Measurement of cell proliferative activity can predict the clinical outcome of malignancy. In previous studies it was measured by mitotic counts as well as flow cytometry. Recently, immunohistochemical staining has become a simple method availing rapid results. As these molecular markers will detect the neoplastic genetic alterations in the cells which could not be detected by H & E stain, they have an important role in diagnosis. Immunohistochemistry is an indispensable tool for diagnostic as well as research purpose in human disease, and is widely employed in establishing diagnosis. It is a method for demonstrating the presence and location of proteins in tissue sections.

To establish tumor free surgical margins, molecular markers have an important role, they detect genetic changes and thereby help in complete resection of the tumor. Appropriate tumor markers have the ability to detect the neoplastic changes in cellular levels, so they can determine precise involvement of cancer in the surgical margins which are stained histopathologically negative which will prevent the high local recurrence rate and may guide to patients for additional chemotherapy or radiotherapy and regular follow up. So rather than histopathology, immunohistochemical staining of surgical margins is a better choice of investigation to diagnose patients with high risk of local recurrence and tumor associated deaths. Immunohistochemical staining is a simple method which gives rapid results, with commercial availability of new antibodies for formalin fixed tissues, making it the technique of choice over other methods. Amongst those most often used, antibodies against “Ki-67 antigen” is a very reliable proliferative marker of choice.

Proliferation of cells is an important biological process in the growth and development as well as regulator of homeostasis of tissue. By assessing cell proliferation with expression of immunohistochemical evaluation disease free survival can be predicted. Thus it is very important to evaluate proliferative status of a tumor to decide its biological aggressiveness.(2)

The cell cycle has G₀, G₁, S, G₂ and M phases, the expression of “Ki-67” is seen in these phases, but is not seen in quiescent G₀ phase. In tissue section, the “Ki-67” antigen is used to localize the “Ki-67” protein (p” Ki-67”) (3). The antibody which is raised against the “Ki-67” antigen is used as a usual, fast and confirmatory means of evaluating the increase of fraction of normal and neoplastic cell populations.

Other important aspect of cell proliferation concerns the rate of cell cycle. This can be evaluated by silver stained or “argyrophilic nucleolar organizer regions”. (AgNORs)

“AgNORs” are structural and functional units of the nucleolus in which all the components necessary for ribosomal RNA synthesis are located. Nucleolar organising regions (NORs) contain ribosomal genes, which encode for ribosomal RNA. A peculiar group of acidic proteins that are highly argyrophilic are also localized at the same sites as NORs, thus allowing NORs to be very clearly and rapidly visualized by silver nitrate staining procedures. The number of interphase AgNORs are strictly related to rRNA transcriptional activity and, in continuously proliferating cells, to the rapidity of cell proliferation.(4) AgNOR and “Ki-67” can provide valuable information about cell proliferation velocity in tumors, and the total fraction of proliferating cells, respectively

The percentage of proliferated cells population at any particular time represents the proliferative state of the cell in terms of the cell biology. The “Ki-67” expression evaluates the proportion of cells committed in the proliferation state of the cell cycle”.Ki-67” protein is used as a growth fraction marker it detects the proportion of cells committed to the cell cycle. Hence it assesses the state of cell proliferation.

The other important aspect of cell proliferation concerns the rate of cell cycle. This can be evaluated by an “argyrophilic nucleolar organizer region (AgNOR)” parameter. Interphase AgNOR are structural and functional units of the nucleolus, in which all of the components necessary for ribosomal RNA synthesis are located. The number of interphase AgNOR are strictly related to rRNA transcriptional activity, and in continuously-proliferating cells, to the rapidity of cell proliferation.(4)

Therefore, the following formula expresses the actual proliferative activity of a lesion . Actual proliferative activity = “Ki-67” or MIB-1 scores x AgNOR quantity In this study we will evaluate actual proliferative activity in the histopathologically negative surgical margins using the “Ki-67” labeling index (LI) and AgNOR count, and its correlation with 2 years recurrence.

“Actual proliferative activity” was calculated by using the following equation.

Ki67 Labeling index:

“ $LI = \frac{\text{Number of Ki67 positive cells}}{\text{Total number of tumor cells observed}} \times 100$ ”

‘Actual Proliferative Index (PI) = “Ki-67” or MIB-1 LI × AgNOR count (5).’

We will evaluate actual proliferation index which is calculated by above formula in histopathologically negative surgical margins which are resected during radical neck dissection of oral squamous cell carcinoma patients.

This is a prospective observational study. The patients detailed history and clinical and histopathological records are collected. H&E staining of surgical margins will be performed.

Immunohistochemical staining by “ki-67,” antibody will be done in the negative histopathological surgical margins. The same sections will be stained with “AgNOR” staining. Both sections will be evaluated.

Actual proliferative index will be calculated with above given formula.

Recurrence of tumor will be observed during first two years period after surgical procedure.

Objectives –

- To evaluate the surgical margins of OSCC by H and E stain.
- To evaluate the staining of AgNOR in surgical margins of OSCC.
- To evaluate the staining of Ki-67 in surgical margins of OSCC.

- To calculate the “actual proliferation index” measured by “expression of Agnor and Ki67” in surgical margins of OSCC.
- To correlate the “actual proliferation index” with recurrence of OSCC.

“Materials and methods:”

Setting–This research will be conducted in the Oral Pathology & Microbiology department, SPDC, DMIMS.

Study design – Prospective observational study

Participants–

Inclusion criteria

1. Histopathologically diagnosed cases of OSCC who are undergoing radical neck dissection .
2. Patient who were on regular follow up.
3. Patients who give consent.

Exclusion criteria

1. OSCC patients who were not undergoing radical neck dissection.
2. Patient who lost to follow up
3. Patients who did not give consent

Sample size: Sample size was calculated using the formula given by statistician. The formula is attached.

77 patients of OSCC are selected.

The present study is a prospective cohort study. OSCC patients who are undergoing surgical radical neck dissection are included in the study.

The results of the research will be fed into “SPSS and analyzed by Chi-square test, t-test, one-way ANOVA, and Scheffé test”. A significant level of $\alpha = 0.05$ was used.

- Clinico-histopathologically diagnosed cases of OSCC are taken for study
- Haematoxylin and Eosinophilic(H & E) staining will be done
- AgNOR and Ki67 immunohistochemistry will be done

H and E stained slides of surgical boundaries of OSCC patients will be observed.

AgNOR and Ki67 stained slides of surgical boundaries of OSCC patients will be observed.

Actual proliferation index will be measured by using above formula:

Variables –

- Outcome – recurrence
- Exposure –OSCC
- Dependent variable – H and E staining , Agnor and Ki67 staining.

Statistical methods :-

“This prospective cross-sectional study included OSCC patients who underwent therapeutic surgical treatment in AVBRH, Sawangi ,Wardha. The Kaplan–Meier method was used to estimate survival outcomes. A multivariable Cox regression model was used to evaluate the associations of various clinicopathologic features with 5-year overall survival (OS) outcomes in patients.”

Discussion:

In spite of modern treatment modalities, of oral squamous cell carcinoma patients survival rate is 64.4% (Damella Zononi et al 2019) and 20.7 % by (Juliet Asioet el al) . Survival rates without recurrence is 92% (Camisasca *et al*), and 79.9%(Wang *et al*), 45-50% (*Markopaulous*) and with Recurrence 30%-31.8% .Causes of death in OSCC are Recurrence , Metastasis, Cachexia , Comorbid illness. Cause of recurrence are regional metastasis and positive surgical margins (Taghavi N. et al 2015) . Monique G. et al 2020 has given the concept of field cancerization which states that, chronic carcinogenic exposures affects the entire superficial epithelium of the exposed tissue and has an increased risk for developing premalignant lesions because of multiple genetic abnormalities .To demonstrate these changes histopathological and IHC markers are utilized . Immunohistochemical evaluation of surgical margins is more reliable method to detect the minimal residual cancer , or genetic alterations and these methods are less expensive in relation to complex genetic analysis and PCR .

The actual proliferative index (API) which is calculated by following formula, demonstrates actual proliferative activity and disease progression .

“Pich A et al”has given API formula to calculate proliferative index in pre malignant and malignant lesions. (“**Actual Proliferative Index (PI)**” = **Ki-67 or MIB-1 LI × AgNOR count**”). It is a very useful prognostic factor,also it helps as promising treatment determining modality for patients with premalignant and malignant lesions(6). **Researchers had studied** API co- relation with epithelial proliferation and disease progression. API can distinguish between dysplastic and non-dysplastic leukoplakia . So , it can be useful to evaluate dysplasia in surgical margins.(5)

Cell proliferative activity which is assessed by using “Ki67” antigen staining in individual OSCC patients provide, distinctive, prognostic state, diagnosis and thereby deciding management . 5years survival rate of oscc patients is higher (93%)with low Ki LI expression. Followed by moderate (46-60%) and low(23%). This indicates that increase expression of Ki-67 corelates with disease progression.(7) Recurrence of oral squamous cell carcinoma occurred in first 2 yrs after treatment. The range of recurrence time period is from 2 to 96 months. Median of 14 months. The recurrence time ranges from 2- 96 months . Mean recurrence time is 14 months . (8). Priya AK D'Cruz PS Paiet al studied the impact of surgical margins on recurrence of OSCC patients. They analyzed status of margins at resection. According to their study, Negative margins ≥ 7.5 mm,Close margins 1.5mm ,Positive margins ≤ 1 mm.(22.2%) in resected specimens. cases with negative margins developed a recurrence as compared t1 (30.4%) cases with close margins and 42.8%) cases with positive margins . According to Chandak et al API in normal mucosa was found to be 7.41,in Leukoplakia - 52.51. In OSCC-180.9. In this study a positive co-relation was noted between APIs and histologically malignancy grading system.(11-14)

Conclusion:

The API measured by using AgNOR and Ki67 expression in surgical margins of OSCC, can predict the recurrence in OSCC patient.

The API measured by using AgNOR and Ki67 expression in surgical margins of OSCC, can predict the survival in OSCC patient.

Routine usage of frozen IHC using “API in the evaluation of surgical margins can give real time information to the surgeons if their tumor resection is sufficient.

IHC on paraffin embedded tissue sections using AgNOR and Ki-67 markers can identify subset of cases with a risk of recurrence and may help in modifying treatment protocol and follow-up visits.

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