Research Article

Association of Ki-67 Proliferation Index with Histopathological Differentiation in Locally Advanced Head and Neck Squamous Cell Carcinoma

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ABSTRACT

INTRODUCTION: Ki-67 is a well known marker of cellular proliferation. Its clinico-pathological importance in locally advanced head and neck squamous cell carcinoma (LAHNSCC) is not clearly defined.

METHODS: This prospective, observational study included histopathologically confirmed 90 patients with LAHNSCC. Biopsy specimens were assessed for Ki-67 expression using immunohistochemistry, and the labelling index was recorded as a continuous variable. The squamous cell cancers were graded as well-differentiated (WDSCC), moderately differentiated (MDSCC), or poorly differentiated (PDSCC). Differences in Ki-67 expression across histopathological grades were analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc testing.

RESULTS: The mean age of the cohort was 57.2 years (range 37–74). Histopathological grading revealed 13 (14.4%) WDSCC, 66 (73.3%) MDSCC, and 11 (12.2%) PDSCC cases. Mean Ki-67 expression increased progressively with worsening differentiation: 6.00% in WDSCC, 23.67% in MDSCC, and 78.36% in PDSCC. One-way ANOVA revealed a significant difference across all the grades ($F(2,87)=54.04$, $p<0.001$). The Post-hoc analysis showed significant differences between all the differentiation categories ($p<0.001$). A weak positive correlation was observed between Ki-67 expression and baseline TLC ($r=0.147$); however, this association did not reach statistical significance ($p=0.168$). Ki-67 expression did not differ significantly according to tonsillar fossa involvement ($p=0.094$).

CONCLUSIONS: Ki-67 labelling index demonstrates a strong quantitative proliferative gradient across histopathological grades in LAHNSCC, with markedly elevated expression in poorly differentiated cancers. These findings reinforce the role of Ki-67 as an objective marker of cancer proliferative activity and histological aggressiveness in advanced-stage disease.

Keywords: Proliferation, Head and Neck, Tonsillar Fossa, Expression

1. INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is one of the leading cancers globally. Every year, approximately 9 lacs new cases and around 4.5 lacs deaths are reported of HNSCC [1]. Its incidence is high in South Asia because of exposure to tobacco and alcohol [2]. HNSCC is treated by combination of surgery, radiotherapy and chemotherapy depending on the stage and various factors. Even after the multi-modality treatment, disease progression and recurrence rate is high. These patients present in locally advanced stage, and they do not respond well to the standard management protocol. So, there is a need for biomarkers that may represent intrinsic cancer biology and may predict variations in cancer behaviour within the similar stage groups [3].

Ki-67 is an established indicator of cellular proliferation. It is a well-known nuclear antigen which is produced in active stages of the cell

cycle [4]. Elevated Ki-67 labelling index is found to be associated with higher grade of the tumor and unfavourable biological behaviour in many solid cancers [5, 6]. Still, clinico-pathological significance of Ki-67 in HNSCC is not fully understood, especially in locally advanced HNSCC [7]. While various studies have demonstrated Ki-67 expression in HNSCC, there is still no quantitative assessment of proliferative patterns in advanced-stage HNSCC [8].

Histopathological grading of differentiation is a key factor of pathological assessment in HNSCC and it serves as a surrogate parameter for biological aggressiveness of cancer. Poorly differentiated cancers show higher mitotic activity, higher genomic instability, and disrupted cell-cycle regulation in comparison to well-differentiated cancers [9, 10]. Systematic quantification of proliferation in various differentiation categories in locally advanced HNSCC has not been deeply explored [11]. Establishing whether Ki-67 expression depicts a reproducible biological gradient across differentiation grades may increase its value in pathological assessment [12].

Cancer behaviour in HNSCC may also differ according to the primary subsite. Oropharyngeal cancers, including those originating from the tonsillar fossa, show distinct biological characteristics compared with non-oropharyngeal cancers [12]. Whether proliferative activity differs meaningfully by subsite within locally advanced disease warrants further evaluation. Therefore, the present study was undertaken to assess the association between Ki-67 labelling index and histopathological differentiation in patients with locally advanced HNSCC. Secondary objectives included evaluation of its relationship with primary tumor subsite and exploratory assessment of its association with baseline inflammatory parameters.

2. MATERIALS AND METHODS

This prospective, observational study included 90 patients diagnosed with locally advanced HNSCC at a tertiary cancer centre. Eligibility criteria included equal to or more than 18 years of age, histo-pathologically confirmed HNSCC and stage III–IVA according to the AJCC 8th edition staging system. Exclusion criteria included non-squamous histology, patients with prior treatment (radiation, chemotherapy, surgery), distant metastasis and recurrent disease. Institutional Ethics Committee clearance was taken and written informed consent was obtained from all participants. Baseline demographic and clinico-pathological parameters, including age, tumor differentiation, primary tumor subsite, and baseline hematological parameters were recorded at diagnosis.

The biopsies were embedded in the paraffin and after that fixed in 10% formalin. They were viewed after accurate staining with Hematoxylin & Eosin stain. The histopathological grading of differentiation was done into three categories as WDSCC (well differentiated squamous cell carcinoma), MDSCC (moderately differentiated squamous cell carcinoma) and PDSCC (poorly differentiated squamous cell carcinoma). Monoclonal anti Ki-67 antibody was used for immunohistochemical staining for Ki-67. Detection was carried out using a standardized secondary antibody system with chromogenic visualization. The Ki-67 labelling index was defined as the percentage of positively stained tumor cell nuclei among at least 1,000 tumor cells counted in representative high power fields (HPF) within areas depicting the highest proliferative activity (“hot spots”). The index was recorded as a continuous variable and used for all statistical analyses. Primary tumor subsites were categorized based on clinical and pathological documentation. For comparative analysis, tumors were stratified according to tonsillar fossa involvement (present vs absent).

2.1 STATISTICAL ANALYSIS

Data was entered into microsoft excel sheet and Python was used for statistical analyses. The normality of continuous variables assessed with the use of Shapiro–Wilk test and they were expressed as mean ± standard deviation (SD). Categorical variables were recorded as percentages and frequencies. Mean Ki-67 labelling index was statistically compared in histopathological grades via one-way analysis of variance (ANOVA). The post-hoc comparisons were done via Tukey’s Honestly Significant

Difference (HSD) test after detecting overall significance. Effect size was calculated using eta squared (η^2), derived from the ANOVA, to quantify the proportion of total variance in Ki-67 expression explained by tumor differentiation. Cohen's f was calculated from η^2 to estimate standardized effect magnitude. Post-hoc statistical power was estimated assuming a two-tailed α of 0.05. Differences in Ki-67 expression between tumors with and without tonsillar fossa involvement were assessed using independent samples t-tests. Pearson correlation analysis was performed to evaluate the association between Ki-67 labelling index and baseline total leukocyte count. The correlation coefficient (r) and corresponding p-value were reported. All statistical tests were two-tailed, and a p-value <0.05 was considered statistically significant.

3. RESULTS

3.1 Patient and Tumor Characteristics

After applying inclusion and exclusion criteria, 90 patients with LAHNSCC were included in the present study. Baseline clinico-pathological characteristics are summarized in Table 1. The mean age was 57.2 years. WDSCC, MDSCC AND PDSCC were 14.4%, 73.3% and 12.2% respectively. Tonsillar fossa was involved in 28.9%. The mean baseline TLC was 9,186 cells/mm³.

Table 1. Baseline clinico-pathological characteristics of patients (n=90)

Variable	Value
Age (years)	57.2 (range 37–74)
Tumor Differentiation, n (%)	
Well-differentiated (WDSCC)	13 (14.4%)
Moderately differentiated (MDSCC)	66 (73.3%)
Poorly differentiated (PDSCC)	11 (12.2%)
Primary Tumor Subsite, n (%)	
Tonsillar fossa involvement	26 (28.9%)
Non-tonsillar sites	64 (71.1%)
Baseline Total Leukocyte Count (cells/mm³)	9,186

3.2 Ki-67 Expression Across Histopathological Grades

The mean Ki-67 labelling index depicted a progressive increase with poor differentiation histology as 6.00% with WDSCC, 23.67% with MDSCC, and 78.36% with PDSCC. There was significant difference in Ki-67 expression amongst the various histopathological grades ($F(2,87) = 54.04$, $p<0.001$). Post-hoc analysis revealed significant differences between WDSCC and MDSCC, MDSCC and PDSCC, and WDSCC and PDSCC (all $p<0.001$). Ki-67 distribution across grades is presented in Table 2 and illustrated in Figure 1.

Table 2. Ki-67 Labelling Index across histopathological grades (n=90)

Histopathological Grade	n	Mean Ki-67 (%)
Well-differentiated (WDSCC)	13	6
Moderately differentiated (MDSCC)	66	23.67
Poorly differentiated (PDSCC)	11	78.36

One-way ANOVA: $F(2,87) = 54.04$, $p < 0.001$
Post hoc (Tukey HSD): All pairwise comparisons significant ($p < 0.001$)
Effect size: $\eta^2 = 0.554$; Cohen's $f = 1.12$

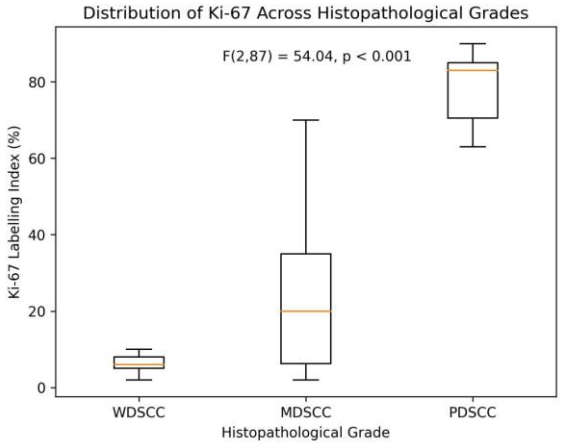


Figure 1. Distribution of Ki-67 Labelling Index across histopathological grades in LAHNSCC

Box-plot illustrating the distribution of Ki-67 labelling index among WDSCC, MDSCC, and PDSCC tumors. A progressive increase in proliferative activity is clearly seen with poor degree of differentiation.

3.3 Effect Size and Power Estimation

Effect size analysis demonstrated a very large association between histopathological grade and Ki-67 expression, with eta squared (η^2) = 0.554, indicating that 55.4% of the total variance in proliferative index was explained by tumor differentiation. The corresponding Cohen's f was 1.12, consistent with an exceptionally large effect size. Post-hoc power analysis ($\alpha = 0.05$)

demonstrated statistical power exceeding 99.9% for detecting differences across the three differentiation groups. These findings confirm that the observed proliferative gradient is not only statistically significant but also quantitatively substantial.

3.4 Ki-67 Expression and Primary Tumor Subsite

Mean Ki-67 expression was compared in HNSCC with and without tonsillar fossa invasion. The mean Ki-67 value was 35.23% in HNSCC with tonsillar fossa invasion (n=26), which was higher in comparison to 24.78% in HNSCC without tonsillar fossa invasion (n=64). But, this difference was not statistically significant (p=0.094). So, primary tumor subsite was not significantly associated with proliferative activity in the present study.

3.5 Correlation Between Ki-67 and Baseline Inflammatory Marker

Pearson correlation analysis demonstrated a weak positive association between Ki-67 expression and baseline total leukocyte count (r=0.147). However, this relationship did not reach statistical significance (p=0.168), suggesting that systemic inflammatory burden is not a primary determinant of tumor proliferative activity in the present study. (Figure 2)

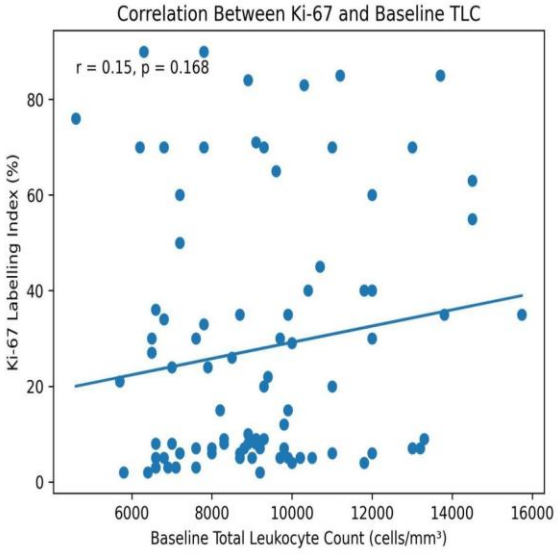


Figure 2. Scatter plot showing the relationship between Ki-67 expression and baseline total leukocyte count.

4. DISCUSSION

The present study provides a quantitative assessment of the Ki-67 proliferation index in locally advanced head and neck squamous cell carcinoma (LAHNSCC), demonstrating a pronounced and progressive biological gradient across various histopathological grades. The findings from the present study confirm that level of Ki-67 expression closely reflects tumor differentiation and proliferative activity in advanced-stage HNSCC. The most significant observation in the present study was the statistically significant progressive increase in mean Ki-67 expression from 6.00% in WDSCC to 23.67% in MDSCC and 78.36% in PDSCC. One way ANOVA demonstrated a highly significant difference across groups ($p<0.001$), with a very large effect size ($\eta^2=0.554$), indicating that more than half of the total variance in Ki-67 proliferative index was explained by histopathological grade.

The increase in proliferative activity of the cancer cells parallels their loss of cellular maturation and architectural organization which is characteristic of dedifferentiation. Well differentiated tumors showed low proliferative activity. In contrast, poorly differentiated tumors showed increased Ki-67 expression, reflecting high mitotic activity and low regulatory control [13]. The reported increase in Ki-67 expression with decreasing differentiation is biologically coherent with cancer biology, where dedifferentiation is linked to disruption of cell-cycle checkpoints and genomic instability [14]. Similar proliferative gradients have been documented in HNSCC which suggest the transition from well-differentiated to poorly differentiated states. Ultimately, there is a fundamental shift towards a more aggressive phenotype [15]. The magnitude of difference observed in the present study, particularly between well- and poorly differentiated tumors, supports the role of Ki-67 as a quantitative parameter to conventional histopathological grading.

HNSCC with tonsillar fossa invasion revealed higher Ki-67 expression vs non-tonsillar sites (not significant, p=0.094). While subsite related biological variation may exist, histopathological differentiation was found as the major determinant of proliferative activity in the present study. The molecular landscape of HPV-

associated oropharyngeal carcinoma is well recognized [16]. Viral oncoproteins such as E6 and E7 disrupt p53 and Rb pathways, promoting deregulated cell-cycle progression. HPV status was not assessed in the present study. The possibility that subsite specific molecular markers influence proliferation, warrants further investigation in studies incorporating p16 or HPV testing.

Pearson correlation analysis depicted a weak positive association between baseline TLC and Ki-67 expression. But this was not statistically significant which indicates that systemic inflammatory burden was not independently associated with tumor proliferative activity in the present study. Inflammation related to cancer is associated with tumor progression via cytokine mediated activation of proliferative signalling pathways, including JAK/STAT and NF-κB cascades [17-19]. Although the direction of association was seen in the present study, but the absence of statistical significance confirms that systemic inflammation may not be a primary driver of tumor cell proliferation in locally advanced HNSCC. Rather, histopathological differentiation appears to show stronger influence on the proliferative activity.

4.1 CLINICAL IMPLICATIONS

The clear quantitative separation in Ki-67 expression across differentiation categories supports its potential utility as an objective adjunct to conventional histopathological assessment [20]. Given that histological grading may be subject to inter-observer variability particularly in moderately differentiated cancers, the incorporation of a standardized proliferative index may enhance diagnostic reproducibility and provide additional biological characterization [21]. Quantitative Ki-67 assessment may be especially informative in borderline cases where morphological features do not unequivocally distinguish between moderate and poor differentiation. In such settings, the proliferative index may function as a complementary biomarker that reflects underlying tumor kinetics rather than relying solely on architectural features [22, 23]. The very large effect size observed in the present study further underscores the biological relevance of proliferative activity as a defining characteristic of tumor aggressiveness [24].

Beyond the utilization in diagnostic refinement, identification of tumors demonstrating disproportionately elevated proliferative indices may contribute to improved biological risk stratification within stage matched groups. Stage matched patients show different clinical outcomes, and integration of proliferation-based markers may help account for this variability. Elevated proliferative activity could reflect enhanced replication and genomic instability, hallmarks commonly associated with aggressive cancer behavior [25]. However, the prognostic implications of Ki-67 in locally advanced HNSCC remain to be definitively established. While the proliferative gradients observed in the present study are biologically compelling, prospective investigations incorporating survival endpoints and molecular profiling are necessary to determine whether Ki-67 independently predicts treatment response, disease recurrence, or overall survival [22, 26].

4.2 LIMITATIONS

Limitations of the present study include single centre study and absence of long term survival outcomes for the different groups. HPV or p16 status was not seen in the present study, hence it limits interpretation of subsite specific biological variation. A weak positive association between systemic inflammation and proliferative index was reported. Multicentre studies including large number of patients with molecular profiling and survival endpoints are needed to evaluate the prognostic significance of the Ki-67 proliferative index in locally advanced HNSCC.

5. **CONCLUSION**

This prospective study concludes that in locally advanced head and neck squamous cell carcinoma, there is a robust and statistically significant correlation between the Ki-67 proliferation index and histological differentiation. From well-differentiated to poorly differentiated tumors, there was a noticeable and progressive rise in Ki-67 expression. The differentiation was found to be responsible for a significant amount of proliferative variability. Ki-67 expression was

not substantially correlated with the main tumor subsite or the initial state of systemic inflammation. These results demonstrate Ki-67's potential use in improving biological characterization and risk stratification in advanced-stage disease and justify its use as an objective, quantitative supplement to traditional grading.

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We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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ETHICAL INFORMATION
Institutional Ethics Committee clearance was taken for this study (via Biomedical Research Ethics Committee no. BREC/22/TH/RTO-02 dated 20.02.2023) and written informed consent was obtained from all participants

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