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ORIGINAL ARTICLE

Pathological and Clinical Analysis of Biomarkers in Head and Neck Squamous Cell Carcinoma (HNSCC): A Diagnostic and Prognostic Study

Heba Mohamed Mahmoud Hamdy[1*] and Mona Saeed Mustafa Mohammed[2]
[1] Laryngology department, Faculty of Medicine, Zagazig University, Zagazig, Egypt.
[2] pathology department, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Coresponding author: Heba Mohamed Mahmoud Hamdy
E-mail: hebaelomda15@gmail.com

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ABSTRACT

Background: Head and neck squamous cell carcinoma (HNSCC) exhibit marked biological heterogeneity in behaviour and prognosis. Immunohistochemical (IHC) markers like Ki67, p53, and p16 can offer valuable information regarding the aggressiveness of tumors and may help identify potential targets for therapy. This study aims to assess the immunohistochemical expression of these markers in head and neck squamous cell carcinoma (HNSCC)." and correlate these findings with clinicopathological parameters.

Methods: This retrospective observational study included 80 histologically confirmed HNSCC cases from Zagazig University Hospitals (2017–2021). Immunohistochemical staining for Ki67, p53, and p16 was performed on formalin-fixed, paraffin-embedded tumour sections. Biomarker expression was assessed independently by two blinded pathologists and analyzed in relation to clinicopathological parameters. Statistical analyses included Chi-square tests, Kaplan–Meier survival analysis, and Cox regression, with significance set at $p < 0.05$.

Results: Among 80 HNSCC patients (mean age 60.3 years; 83.8% male), the larynx was the most common tumour site. High Ki67 expression, observed in 34% of cases, was significantly associated with poor differentiation, advanced stage, and shortened overall survival. Mutant p53expresion was detected in 55% and correlated with poor differentiation, nodal metastasis, and reduced disease-free survival. p16 positivity (18.8%) was strongly associated with oropharyngeal tumors, early stage, and improved survival outcomes. Multivariate analysis identified high Ki67, mutant p53, and p16 positivity as independent prognostic factors.

Conclusion: Ki67 and mutant p53 are associated with aggressive tumour behaviour. p16 expression, though infrequent, suggests a favourable prognosis. Combined biomarker analysis enhances prognostic stratification.

Keywords: Ki67, P53, P16, Larynx, Oropharynx.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) comprises a diverse group of epithelial malignancies that arise from the mucosal surfaces of the oral cavity, pharynx, and larynx Globally, it is the sixth most prevalent cancer, accounting for around 890,000 new cases and approximately 450,000 deaths each year. Despite advancements in surgical interventions, radiotherapy, and chemotherapy, the overall five-year survival rate remains unsatisfactory, particularly in advanced stages. This is largely due to late diagnosis, high recurrence rates, and the tendency for metastasis.[1].

A major challenge in managing HNSCC is its considerable biological variability. The TNM staging system is widely used to determine disease extent, but it does not account for molecular differences that influence tumour

behaviour, treatment response, and prognosis. Consequently, there is growing interest in identifying and integrating molecular biomarkers into routine assessment to refine risk stratification and guide personalized therapy.[2].

Biomarkers, which are measurable indicators of biological conditions, can have diagnostic, prognostic, or predictive roles. In HNSCC, three biomarkers have been extensively studied: Ki67, p16, and p53. **Ki67** is a nuclear protein associated with cellular proliferation; its over expression is often linked to higher tumour grade, increased aggressiveness, and poorer survival, making it a valuable proliferation index.[3].

The tumour suppressor protein **p16** serves multiple functions. It is notably over expressed in HPV-associated oropharyngeal squamous cell carcinomas, where it is generally linked to a better prognosis. However, its prognostic value in HPV-negative HNSCC remains less clear and may depend on tumour location and other pathological features.[4].

p53, commonly known as the "guardian of the genome," is one of the most frequently mutated genes in head and neck squamous cell carcinoma (HNSCC). These mutations lead to uncontrolled cell proliferation, resistance to apoptosis, and defective DNA repair mechanisms. These alterations are typically associated with a worse prognosis and reduced response to therapy. In contrast, tumors expressing wild-type p53 may be more responsive to treatment and linked to more favourable outcomes.[4].

Integrating biomarker evaluation—particularly Ki67, p16, and p53—into clinical practice can enhance diagnostic precision and provide valuable prognostic information. By analyzing the expression patterns of Ki67, p16, and p53 alongside clinical and pathological variables, healthcare providers can more accurately stratify patients and optimize treatment plans. This approach aligns with the principles of precision medicine, where molecular profiling plays a crucial role in guiding therapeutic decisions beyond traditional anatomical staging.[3].

METHODS

This retrospective observational study with a minimum follow-up period of 36 months was conducted on 80 histologically confirmed HNSCC patients who underwent surgical resection at Zagazig University Hospitals between 2017 and 2021. All patients included had primary, untreated, resectable HNSCC. Patients with recurrent disease or prior chemo/radiotherapy were excluded to eliminate confounding treatment effects.

Tumors were staged according to the 8th edition of the AJCC TNM staging system (2017), and classified histologically according to the 2022 WHO Classification of Head and Neck Tumors. Demographic and clinical data including age, sex, tumour location, TNM stage, histological grade, and survival outcomes were retrieved.

Formalin-fixed, paraffin-embedded (FFPE) tumour tissue blocks were retrieved. Blocks sectioned into 4-μm thick slices and stained with standard H&E staining. Standard H&E confirmed diagnosis. Sections of 3-μm thickness were prepared and subjected to immunohistochemical staining for Ki67, p53, and p16. Heat-induced epitope retrieval was performed using citrate buffer. The sections were incubated with primary antibodies: Ki67 (clone MIB-1, Dako), p53 (clone DO-7, Dako), and p16 (clone E6H4, Ventana). Detection was accomplished using a polymer-based detection system and DAB chromogen, followed by haematoxylin counterstaining.

Staining evaluation was performed by two independent pathologists blinded to clinical data. Ki67 expression was categorized as low (<10%), moderate (10–50%), or high (>50%) nuclear positivity [5,6]. p53 staining was interpreted as mutant type when there was strong uniform positivity in >90% of tumour cells or complete absence of staining, and as wild-type when variable staining was observed in <50% of cells [7,8]. p16 was considered positive when there was strong nuclear and

cytoplasmic staining in ≥70% of tumour cells [9,10].

Ethical code approval: **ZU-IRB #1428 3/6-2025.**

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS software version 26. Associations between biomarker expression and clinicopathological variables were evaluated using Chi-square and Fisher's exact tests. Survival analysis was performed using Kaplan–Meier curves and log-rank tests. Cox proportional hazards regression was applied to determine independent prognostic factors. A p-value less than 0.05 was considered statistically significant.

RESULTS

The study included 80 patients, aged between 39 and 82 years, with a mean age of 60.3 ± 9.4 years. The majority were male (67 patients, 83.8%), while females represented 16.2% (13 patients). The most common tumour site was the larynx (45%), followed by the oropharynx (30%) and oral cavity (25%).

Classification into laryngeal and extra-laryngeal groups revealed significant differences in marker expression, reinforcing their diagnostic value.

Histologically, 80% of the cases were classic squamous cell carcinoma. The remaining cases included basaloid carcinoma in 10%, verrucous carcinoma in 6.25%, and mixed histological sub-types in 3.75% of patients. Tumour grading showed that 12.5% were well-differentiated (G1), 52.5% were moderately differentiated (G2), and 35% were poorly differentiated (G3).

Ki67 expression varied across the cohort. Low Ki67 labeling (<10%) was observed in 16 cases (20%), moderate expression (10–50%) in 37 cases (46%), and high expression (>50%) in 27 cases (34%). High Ki67 expression was significantly associated with poor tumour differentiation (G3, p = 0.002), advanced clinical stage (p = 0.01), and decreased overall survival (OS) (p = 0.004)**.**

p53 expression was mutant-type in 44 cases (55%) and wild-type in 36 cases (45%). The presence of mutant p53 correlated significantly with poorly differentiated tumors (p = 0.01) and lymph node metastasis (p = 0.03). Moreover, patients with mutant p53 had a significantly lower disease-free survival (DFS) compared to those with wild-type expression (p = 0.02).

p16 was positively expressed in 15 cases (18.8%). This expression was predominantly observed in oropharyngeal tumors and showed a statistically significant association with early tumour stage (p = 0.04). Notably, p16 positivity was significantly associated with improved disease-free survival (p = 0.04). **(Table 1,2)**

High Ki67 expression was common in G3 tumors, aiding histological grading. Mutant p53 was linked to aggressive phenotypes. p16 was specific to oropharyngeal SCC, supporting HPV-related diagnosis.

The survival analysis showed a 3-year overall survival rate of 67.5% and a disease-free survival rate of 60%. Better survival outcomes were observed in patients with p16-positive tumors, low Ki67 expression, and wild-type p53 expression. Multivariate Cox regression analysis revealed that elevated Ki67 expression (HR 2.6, p = 0.003), presence of mutant p53 (HR 1.9, p = 0.02), and p16 positivity (HR 0.45, p = 0.046) were independent prognostic markers. **(Table 3) (Figure 1,2)**.

Table 1: Clinicopathological Features and Correlation with Biomarker Expression

Feature	Category	Cases (n)	Ki67 (p-value)	p53 (p-value)	p16 (p-value)	OS Impact	DFS Impact
Age	<60 / ≥60	40 / 40	0.22	0.14	0.40	-	-
Gender	Male / Female	67 / 13	0.33	0.12	0.28	-	-
Tumor Site	Laryngeal / Extra-laryngeal	45 / 35	0.01	0.18	0.003	Yes	Yes
Histological Type	Classic / Others	64 / 16	0.05	0.03	0.08	Yes	Yes

Feature	Category	Cases (n)	Ki67 (p-value)	p53 (p-value)	p16 (p-value)	OS Impact	DFS Impact
Grade	G1 / G2 / G3	10 / 42 / 28	0.002	0.01	0.09	Yes	Yes
Stage	Early / Advanced	30 / 50	0.01	0.04	0.04	Yes	Yes

Table 2: Ki67, p53 and p16 Expression and Clinical Correlations

Marker	Category	Cases (n)	Percentage (%)	Correlation
Ki67 Labeling Index	<10% (Low)	16	20.0	Associated with better OS
	10–50% (Moderate)	37	46.0	Better DFS
	>50% (High)	27	34.0	G3 tumors (p = 0.002), advanced stage (p = 0.01), decreased OS (p = 0.004)
p53 Expression Type	Wild-type	36	45.0	Better DFS
	Mutant-type	44	55.0	G3 tumors (p = 0.01), LN metastasis (p = 0.03), lower DFS (p = 0.02)
p16 Status	Positive	15	18.8	Oropharyngeal tumors, early stage (p = 0.04), improved DFS (p = 0.04)
	Negative	65	81.2	

Table 3: Survival Outcomes and Prognostic Indicators

Outcome / Marker	Value
3-year OS	67.5%
3-year DFS	60%
Better prognosis	p16+, low Ki67, wild-type p53
High Ki67	HR 2.6, p = 0.003
Mutant p53	HR 1.9, p = 0.02
p16 positivity	HR 0.45, p = 0.046

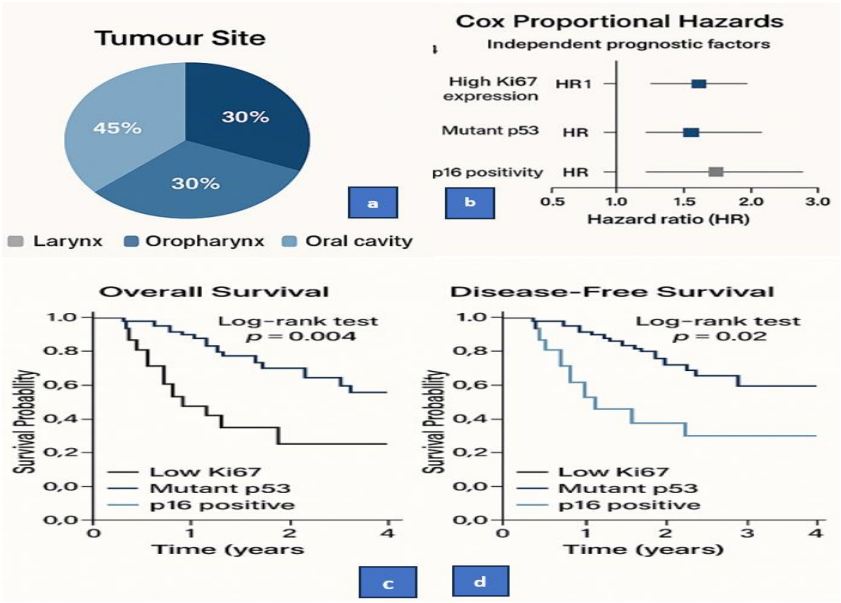

Figure 1: a Pie chart of tumor site distribution. **b** Cox proportional hazards ratios. **C,d** Survival analysis using log-rank tests.

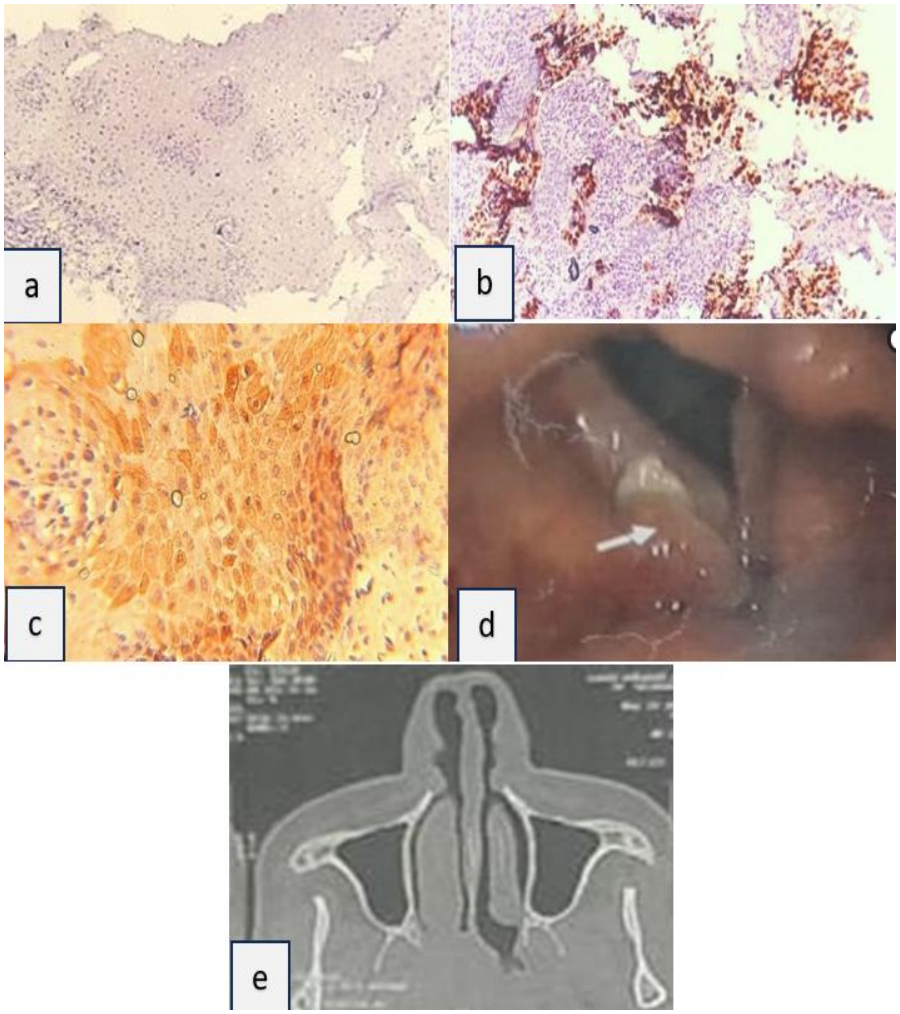

Figure 2: a Negative ki67 immunohistochemical staining in well differentiated squamous cell carcinoma (×20). **b** p53 mutant type immunohistochemical nuclear reaction with variable intensity in some of the tumour cells (×20). **c** Positive p16 immunohistochemical staining in well differentiated squamous cell carcinoma (×40). **d** laryngoscopic examination showed right supraglottic mass. **e** CT coronal view of nasal sinuses showing right nasopharyngeal mass.

DISCUSSION

Our findings corroborate earlier studies indicating Ki67 and p53 as markers of tumour aggressiveness and poor prognosis. High Ki67 expression reflects increased tumour cell turnover and is linked to poor survival. In addition to prognostic significance, these biomarkers aid diagnosis: Ki67 helps grade tumors; p53 subtype analysis distinguishes aggressive forms; p16 confirms HPV-related oropharyngeal origin.

Mutant p53, frequently resulting from TP53 mutations, is associated with genomic instability and resistance to apoptosis. This may lead to uncontrolled proliferation and therapeutic resistance in HNSCC. Earlier studies by **Soussi and Wiman** [7], as well as **Olivier et al.** [8], emphasized the dual impact of p53 mutations in both tumor progression and resistance to therapy, supporting the conclusions of our study.

p16 over expression was uncommon but linked to favorable outcomes, especially in oropharyngeal SCC. This aligns with findings that p16 is a surrogate marker for HPV, conferring better prognosis and treatment response. Therefore, p16 positivity may help stratify patients for DE-escalated therapy. **Lewis et al.** [9] and **Bishop et al.** [10] have previously demonstrated that p16 positivity is independently associated with longer survival and response to radiation-based treatments.

The gender disparity (male predominance) and age distribution were consistent with global trends in HNSCC. [11]. Our multivariate analysis further confirms the prognostic independence of all three biomarkers and supports their inclusion in routine diagnostic evaluation.

Interestingly, the distribution of Ki67 expression in our cohort (20% low, 46% moderate, 34% high) highlights a substantial fraction of tumors with elevated proliferation indices. This aligns with literature noting Ki67's correlation with aggressive histological features, poor differentiation, and reduced response to therapy [12,13]. **Urruticoechea et al.** [5] emphasized the clinical relevance of Ki67 as a reliable marker of cancer proliferation, consistent with our observation.

The predominance of mutant p53 expression (55%) echoes previous reports where alterations in p53 were significantly higher in advanced and poorly differentiated tumors [14,15]. Such mutations are known to result in inactive p53 protein accumulation, which can be detected via immunohistochemistry. The detection approach used in our study was based on guidelines validated in earlier studies by **Danos et al.** [16] and **Puzzo et al.** [17].

Although only 18.8% of cases were p16-positive, this subset had notably better disease-free survival. As reported by **Ang et al.** [18] and **Shi et al.** [19], p16 is strongly associated with HPV-related oropharyngeal carcinomas and may serve as a biomarker for improved therapeutic response.

The use of multiple biomarkers in HNSCC may enhance diagnostic and prognostic accuracy. The combined analysis of Ki67, p53, and p16 expression offers a comprehensive insight into tumour biology and potential therapeutic responses. This integrative approach supports personalized medicine and highlights the value of biomarker-driven classification in guiding treatment decisions, as supported by **Frejborg et al.** [20] and **Hashmi et al**. [21].

This study reinforces the idea that integrating multiple biomarkers (Ki67, p53, and p16) provides a more nuanced risk assessment [3]. Future research should explore the additive prognostic value of combining these markers with genomic and transcriptomic profiling in larger prospective cohorts. Additionally, exploring interactions between these markers and treatment modalities, such as immunotherapy or targeted therapy, may unveil novel therapeutic avenues.

CONCLUSION

Immunohistochemical analysis of Ki67, p53, and p16 provides meaningful prognostic insights in HNSCC. High Ki67 and mutant p53 expressions are associated with poor outcomes, while p16 positivity may predict improved prognosis. Routine biomarker profiling is recommended for risk stratification.

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