ORIGINAL RESEARCH PAPER

Volume-8 | Issue-10 | October - 2019 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr

INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

IMMUNOHISTOCHEMICAL STUDY OF CELL-CYCLE AND NUCLEAR PROLIFERATION MARKERS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

Pathology

MS Tevatia M.D., Consultant & HOD, Dept of Pathology, Command Hospital, Pune

Prabhashankar Mishra* M.D., Associate Professor, Dept of Pathology, Command Hospital, Pune *Corresponding Author

Vikas Gupta M.S., Assistant Professor, Dept of ENT, Command Hospital, Pune

ABSTRACT

Aim: This study aims to find out association between expressions of a panel of two immunohistochemistry (IHC) markers including Cyclin D1 & Ki 67 in cases of head & neck squamous cell carcinoma (HNSCC) in a pan-India cohort.

Materials and methods: A total of 103 cases of HNSCC presenting to our centre between Jan 2016-Dec 2018 with available clinical details were included in the study. Cases of in-situ carcinoma were excluded from the study. All 103 cases were subjected to IHC with Cyclin D1 and Ki 67 following standard protocol by manual method. Cases were then interpreted as positive, negative or indeterminate for the above antibodies.

Results: Ki 67 nuclear proliferation index as a percentage value was found to be associated with higher grade HNSCC and more aggressive clinical behavior of the tumor. Whereas, Cyclin D1 did not show significant association with important tumor attributes.

Conclusion: Ki 67 mitotic index is significantly associated high grade HNSCC. Though, similar findings have been found in many other studies across the world, more such studies from India need to be done to make this finding clinically relevant.

KEYWORDS

INTRODUCTION

HNSCC contributes to roughly one third of all newly detected malignancies in South Asian countries including India, Sri Lanka, Bangladesh and Pakistan [1, 2]. A small number of cases of HNSCC presents at an early stage without any evidence of nodal or distant metastasis, especially so in India [2]. The majority presents with advanced disease where treatment options are limited and prognosis poor. A reliable predictor of aggressive disease in these cases has still not been found.

HNSCC, like most other malignancies, arises from multistep accumulation of numerous genetic aberrations in the basal keratinocytes. The diversity of these genetic events is responsible for widely varying clinical response to treatment in different stage-matched patients of this disease. [3,4]. Morphologic histopathology remains the gold standard for diagnosis of these cases and further adjunct studies like IHC or molecular studies are seldom asked for. In a resource poor country like India, the large burden of disease and poor socio-economic strata of the patients are important forbidding factors. However, recent years have seen increasing use of IHC which suitably complements histopathological analysis by detecting genetic aberrations at the protein level thus providing an affordable surrogate for molecular changes. An immunohistochemical panel using multiple molecular biomarkers can provide useful information about clinico-biologic attributes of HNSCC thereby, helping in management and prognosis of cases. Interestingly, multiple combinations of IHC antibodies have been studied in HNSCC in different parts of the world deriving varied conclusions but clinical application of IHC markers is yet not there on the anvil.

Candidate biomarkers do exist. Cyclin D1 is an oncoprotein which is a critical driver of cell cycle progression, and the vital decision for cell growth or arrest may depend on the nuclear concentration of Cyclin D17[4]. It is widely used as an IHC marker in diagnosis of Mantle cell lymphoma and as a predictive marker in multiple myeloma and estrogen receptor (ER) positive breast cancer. It has also been found to be over expressed in 50-60% of HNSCC though its role as a marker to aid treatment or prognosis is not well established. MIB1 (Ki67) is a proliferation marker expressed by nuclei of cells that have entered the cell cycle. It is widely used in ER positive breast cancer and neuroendocrine tumors but its prognostic role in HNSCC remains controversial [4]. Though, Ki 67 is frequently being used in many different types of malignancies for both diagnosis and grading of the disease, its role in HNSCC needs to be further investigated.

The primary objective of this study is to find out expressions of a panel of IHC markers i.e. Cyclin D1& Ki67 by the tumor cells in cases of HNSCC. Further, any association between their expression and aggressive biological behavior of tumor in these cases will also be studied. This may help in therapeutic decisions and prognostication in an overwhelming number of HNSCC cases especially in countries with high disease burden. Molecular studies are available at limited centres in India and are expensive for most of the poor patient population.

MATERIALS AND METHODS

Patients

All consecutive cases of head & neck squamous cells carcinoma diagnosed and treated at our center between January 2016 and December 2018 with available clinical details and archived biopsy or resection tissues were considered for inclusion in this study. It is important to note here that these patients came from different states and regions of India making it a pan-India representation. A total of 103 cases with adequate archival tissue were finally included in our study. Diagnoses were confirmed by hematoxylin and eosin (H& E) stained sections by experienced pathologists at this center. Tumor Node Metastasis (TNM) stage was done based on clinical examination and radiological evaluation of the case. All cases were treatment naïve and the primary treatment consisted of surgical resection of the tumor with or without nodal dissection and followed by radiation and or chemotherapy in selected patients. Clinical outcome was retrospectively traced from the hospital's records in all cases. Metastatic disease at presentation was defined as regional lymph nodal spread and or spread to another organ in the body. Local contiguous extension of the tumor in surrounding soft tissue of the oral cavity or invasion of the underlying bone was not considered as metastasis. This exercise was done to understand and categorize the tumors as highly aggressive and less aggressive respectively, in cases with advanced and limited clinical disease. Moreover, the clinical outcomes in these cases were followed up for an average of 18 months, though the same was not statistically analyzed for this study. This is so because this study is a part of an ongoing multivariate analysis in similar cases with a larger cohort and over a longer duration of time.

Selection of cases for IHC study

The representative tumor tissue in all 103 cases were selected for IHC with Ki 67 & Cyclin D1 antibodies by manual method.

Antibodies

Monoclonal primary antibodies used in this study were Dako, USA, mouse antibody concentrates. All the antibodies were diluted in phosphate-buffered saline (PBS) and their concentration was titrated to optimal staining as per a previous institutional standardization. All antibody clones were of the mouse or rabbit antihuman immunoglobulin G (IgG) subtype.

Immunohistochemistry

IHC was performed at our centre by manual method as under:

1. Tissues were fixed in 10% formol saline, embedded in laboratory grade paraffin, and cut into 3 -4 µm sections.
2. Deparaffinization was performed with xylene and rehydration done with descending concentrations of laboratory grade alcohol.
3. Tissue antigens were retrieved by boiling in 0.01% citric acid at pH 7.0-8.0 in a microwave oven for 6-8 minutes.
4. Hydrogen peroxide (0.3%) in PBS was used to block endogenous peroxidase activity. Samples were washed, and nonspecific binding was blocked with 1.5% normal serum in PBS for 60 minutes.
5. Primary antibody as mentioned above was added to the samples and incubated at room temperature for 60 minutes.
6. Tissue slides were finally washed in PBS, and secondary antibody (Elite anti-mouse IgG ABC kit; Vector Laboratories, Burlingame, CA) was used according to the manufacturer's recommendations.
7. Diaminobenzidine (DAB) was used for 3 minutes at room temperature for visualization, and Mayer's hematoxylin was used for the background counterstaining.

Microscopic Evaluation

1. All H& E and IHC slides were evaluated by a single blind approach by two independent observers (MST & PS) under Olympus BX- 40 (Olympus, Tokyo, Japan) microscope. In cases with difference of opinions, the slides were reviewed together until a consensus was reached between the two observers.
2. On histopathologic evaluation cases were assigned to well, moderately and poorly differentiated category based on conventional criteria (a mix of Broder's and Anneroth's criteria).
3. A total of 103 tumor slides from an equal no of patients were evaluated for IHC with anti-Ki 67& Cyclin D1 antibodies as per method elucidated above.
4. IHC staining results for both antibodies were evaluated by standard method as described in the literature and cases were termed 'positive', 'negative' or 'indeterminate or non-contributory' respectively for that antibody. Ki 67 index was calculated by counting at least 200 cells in the highest proliferating areas of tumors.
5. A high mitotic index (Ki 67 index) was taken as >20% of tumor cells showing nuclear positivity for this stain.
6. Positivity of any of the above IHC stains was defined as diffuse (>50% cells) and at least moderately intense staining of tumor cells in their respective parts of the tumor cells as described in standard textbooks.
7. Appropriate positive and negative control tissues were put for comparison with every batch of IHC stain.

Recording of data

Associations between IHC marker expression and tumor category were meticulously recorded for further interpretation and finalization of results. Important clinical and pathologic variables which were also recorded in the study included; age, sex, smoking history, tumor location grade of the tumor and depth of tumor invasion.

RESULTS

Out of the total 103 cases, 36 were females with a male to female ratio of 2.9:1. A history of tobacco use was present in 64(65.1%) cases. Twenty-one (20.2%), 52 (51%) & 30 (28.8%) cases were assigned to well, moderately and poorly differentiated grades, respectively. Definitive follow up records were available in 69 cases; 41(40.6%) of these had died or were lost to follow up during this study, 28(27.4%) cases showed recurrence or metastasis after the initial diagnosis and treatment. Various important clinical and pathological features observed and noted as seen in this study are presented in Table1.

Thirty-nine out of total 103 cases (38.8%) revealed a Ki67 mitotic index of more than 20% (Fig1, Table 2).

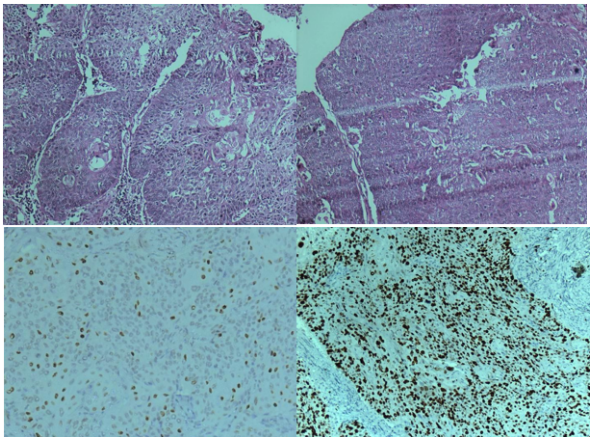
Table 1

Parameter	N=103	
Mean Age	49 Yr	
Sex	M F	67 36
Tobacco use	Never Past Present	31 24 48
Metastatic Disease	Present Absent	46 57
Grade	Well differentiated Moderately differentiated Poorly differentiated	21 52 30
Tumor Location	Lip Buccal mucosa Tongue RMT Others	17 40 33 09 04
Depth of invasion	≤1 cm >1cm	44 59

Tabular details of important clinical and pathological properties of the tumors.

On IHC evaluation of 103 cases 27, 53 and 23 cases were respectively showing a Ki 67 % index of >5, 10 & 20 respectively finally depicting significant association with well, moderate and poor differentiation of tumors as seen on microscopic evaluation representing a p value of ($p<0.001$, CI=0.95) in all three categories .Cyclin D1 , however did not show any notable association with either the grade or stage of the tumors. Other markers including bcl2,C-KIT & HER2/Neu which were also done in these cases also did not show any association with any important clinical or pathological properties of the tumor in all these cases.

Figure1



Hematoxylin and eosin (H&E) photomicrographs showing A; 10X view of a moderately differentiated squamous cell carcinoma; B; 10 X view of poorly differentiated squamous cell carcinoma respectively. C; IHC staining with Ki67 (MIB1) antibody showing a low mitotic index (<20%) in a well differentiated SCC. D; High mitotic index (>20%) with Ki 67 in a high-grade SCC.

Table 2

Tumor Grade	Number of Cases	Ki 67 Index
Well Differentiated	21	27(< 5%)
Moderately Differentiated	52	37 (5-20%)
Poorly Diffrentiated	30	39 (>20%)

IHC evaluation results of 103 cases with anti-Ki 67 antibody

DISCUSSION:

HNSCC is a widely prevalent malignancy and poses a public health problem in many developing countries including India. The situation in India is even worse due to prevalence of tobacco abuse, paan and areca nut chewing as well as bidi smoking. The majority of patients presents at an advanced stage, leading to increased mortality and morbidity. Scarcity of efficient healthcare facilities for early diagnosis and management adds to the increasing incidences of this tumor in India. Public ignorance and socio-economic factors and cultural complexities of this country all aid in perpetuation of this burgeoning problem.

HNSCC is generally a disease of the late adulthood and the elderly, detection of sporadic cases in childhood are extremely rare. The average age of cases in our study is 49 years which is in line with most similar studies from various parts of the world. Tobacco use was reported in approximately 70% cases in this study which only reinforces the role of tobacco in possible etiological association with

this cancer. Other clinical and pathological findings as depicted in Table 1 are similar to findings reported by numerous researchers in their studies and therefore do not require further discussion for the sake of avoiding repetition of well known facts and brevity.

The quest for a widely available and a clinically reliable predictor of prognosis in HNSCC has largely remained elusive. Numerous IHC and molecular markers have been studied in this context with HNSCC. But, none has found favor with the clinicians and therefore this quest still remains unfulfilled and ongoing [2, 7]. Most researchers have included a single or a few IHC markers in their study. We have tried to include a set of commonly available IHC markers and studied them in context with grade and biological aggressiveness of this tumor which by inference are responsible for overall survival and responsiveness of tumors to various modalities of treatment.

Cyclin D1 is a cell proliferation marker which has been studied by various sceintists and researchers in HNSCC [4, 6]. Shikari et al found a significant relation between the adverse overall survival (OS) in oral SCC and over-expression of cyclin D1 along with p53 protein expression in their study [13]. Jayasurya et al reported a similar finding when cyclin D1over-expression coupled with reduced IHC expression of p16 were evaluated in a cohort of oral SCC patients [5,14]. In our study, however, we could not find any association between IHC expression of cyclin D1 and HNSCC. Molecular and genetic studies some of which are also soon to find clinical and laboratory application have unraveled many of the etio-pathogenetic underpinnings of malignancies. However, most of these studies have been done in the affluent western countries. Especially with respect to HNSCC, which is far more common in relatively resource limited countries like ours, the results of such studies have not translated into clinical utility, limiting their usefulness. Moreover, owing to a number of genetic and environmental factors which lie at the heart of carcinogenesis, the findings of similar studies in this part of the world might differ from those in previous studies. This may partially explain disparate findings with respect to Cyclin D1 in our study as compared to those under reference.

Ki 67 is an antibody used to detect a complex of nuclear proteins in cells under synthetic or S phase of cell division and has been strongly associated with cell proliferation. It is not found to be expressed by cells in G0 phase of the cell cycle. Most of the studies including one by Myoung et al have found an association between adverse prognosis and IHC over-expression of Ki 67 in SCC of the oral cavity in their cohort of patients [8, 16]. Lee et al, on the other hand, reported no independent association between its IHC expression and survival in oral SCC cases [9, 10].In this study we found a significant association between grade of the HNSCC at presentation and over-expression of Ki 67 (p value <0.001, CI 95%). Also, other important clinical attributes of the tumors including nodal and distant metastasis were found to be strongly associated with the level of expression of Ki 67 in these cases.This only reiterates the belief that highly proliferating tumors are likely to be more aggressive and possess higher propensity to metastasize and therefore are likely to show markers of high mitosis. Recent strides made in the field of molecular dianostics and research of oncology have proven beyond doubt that mitosis is not just a function or depictor of high tumor turn-over, but it also predisposes the tumor clones to undergo frequent mutations and provides them with vital survival and potential metastatic advantages. Thus, rendering the patients of such tumors with a potential disadvantage in terms of overall poorer clinical outcomes. As we found significant association between Ki 67 index and important tumor properties like tumor grade, nodal and distant metastasis ,this becomes an important finding of our study because of the potential for use of this widely available IHC marker in predicting metastasis in HNSCC cases.This can also be of immense value in therapeutic decision making as well as better prognostication of the cases. Similar findings are reported by most of the studies so far in the literature. One of the important limitations of our study is non-inclusion of IHC for p16 protein expression in these tumors. The author/s is presently evaluating p16 protein over-expression in HNSCC in conjunction with a set of common IHC markers and the results and interpretation of the findings are under evaluation. Further similar studies are required to be undertaken in order to conclusively establish role of this IHC marker in HNSCC.

CONCLUSION:

Based on the findings in this study,the authors conclude that Ki 67 (MIB1) mitotic index by IHC is significantly associated with the tumor grade and biological aggressiveness in cases of HNSCC. This finding may be used as a surrogate marker for prognostication of cases as well as in deciding treatment modalities at the time of presentation. However, further similar studies especially from high disease burden areas need to irrefutably establish its role for it to find useful clinical application.

Acknowledgements: The authors deeply acknowledge PK Reddy, AK Ghosh, Satish Kumar and Nazeem for their tireless and meticulous technical work in carrying out IHC in this study.

Compliance with Ethical Standards:
Funding: This study did not receive any funding from any agency.

Conflict of interest: All authors declare that they have no conflict of interest.

Ethical Approval: No animals or humans were involved in this study. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

REFERENCES:

1. L. R. Oliveira, A. Ribeiro-Silva. Prognostic significance of immunohistochemical biomarkers in oral Squamous cell carcinoma. Int. J. Oral Maxillofacial Surg 2011; 40: 298–307
2. Ferlay J, Shin H-R, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893–2917.
3. de Vicente JC, Lequerica-Ferna´ndez P, Santamarı´a J, Fresno MF. Expression of MMP-7 and MT1-MMP in oral squamous cell carcinoma as predictive indicator for tumor invasion and prognosis. J Oral Pathol Med 2007: 36: 415–424.
4. Oliveira LR, Ribeiro-Silva A, Zucoloto S. Prognostic impact of p53 and p63 immunoexpression in oral squamous cell carcinoma. J Oral Pathol Med 2007: 36: 191–197.
5. Oliveira LR, Ribeiro-Silva A, Costa JP, Simo˜es AL, Matteo MA, Zucoloto S. Prognostic factors and survival analysis in a sample of oral squamous cell carcinoma patients. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008: 106: 685–695.
6. Weigel MT, Dowsett M. Current and emerging biomarkers in breast cancer: prognosis and prediction. Endocr Relat Cancer 2010;17:R245–R262.
7. Xia W, Lau YK, Zhang HZ, Liu AR, Li L, Kiyokawa N, Clayman GL, et al. Strong correlation between c-erB-2 over expression and overall survival of patients with oral squamous cell carcinoma. Clin Cancer Res 1997;3:3–9.
8. Werkmeister R, Brandt B, Joos U. Clinical relevance of erbB-1 and -2 oncogenes in oral carcinomas. Oral Oncol 2000; 32:100–5.
9. Schliephake H. Prognostic relevance of molecular markers of oral cancer— a review. Int J Oral Maxillofac Surg 2003;32:233–245.
10. Shintani S, Nakahara Y, Li C, Mihara M, Nakashiro KI. HER2/ neu expression in oral squamous cell carcinoma. Asian J Oral Maxillofac Surg 2004; 16:172–6.
11. Gonza´lez-Moles MA, Gil-Montoya JA, Ruiz-Avila I, Esteban F, Delgado- Rodriguez M, Bascones-Martinez A. Prognostic significance of p21WAF1/CIP1, p16INK4a and CD44s in tongue cancer. Oncol Rep 2007: 18: 389–396.
12. Jayasurya R, Sathyan KM, Lakshminarayanan K, Abraham T, Nalinakumari KR, Abraham EK, Nair MK, Kannan S. Phenotypic alterations in Rb pathway have more prognostic influence than p53 pathway proteins in oral carcinoma. Mod Pathol 2005: 18: 1056–1066.
13. Shiraki M, Odajima T, Ikeda T, SasakiA, Satoh M,Yamaguchi A,Noguchi M,Nagai I, Hiratsuka H. Combined expressionof p53, cyclin D1 and epidermalgrowth factor receptor improves estimationof prognostic in curatively resected oral cancer. Mod Pathol 2005: 18: 1482–1489.
14. Myoung H, Kim MJ, Lee JH, Ok YJ, Paeng JY, Yun PY. Correlation of proliferative markers (Ki-67 and PCNA) with survival and lymph node metastasis in oral squamous cell carcinoma: a clinical and histopathological analysis of 113 patients. Int J Oral Maxillofac Surg 2006:35: 1005–1010.
15. Lee JI, Jin BH, Kim MA, Yoon HJ, Hong SP, Hong SD. Prognostic significance of CXCR-4 expression in oral Squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009: 107:678–684.
16. Agra IM, Carvalho AL, Pinto CA, Martins EP, Filho JG, Soares FA Kowalski LP. Biological markers and prognostic in recurrent oral cancer after salvage surgery. Arch Otolaryngol Head Neck Surg 2008: 134: 743–749.
17. Diniz-Freitas M, Garcı´a-Caballero T, Antu´ nez-Lo´pez J, Ga´ndara-Rey JM, Garcı´a-Garcı´a . A. Pharmacodiagnostic evaluation of EGFR expression in oral squamous cell carcinoma. Oral Dis 2007: 13: 285–290.