Review Article

Role of proliferative markers in assessing recurrences in surgical margins of oral squamous cell carcinoma: A systematic review

Madhuri Gawande, Samiha Khan, Nidhi Sharma[1], Alka Hande, Swati Patil

Department of Oral Pathology and Microbiology, Sharad Pawar Dental College and Hospital, DMIHER, Sawangi (M), Wardha, Maharashtra, [1]Department of Oral Pathology and Microbiology, Government Dental College, Raipur, Chhattisgarh, India

Abstract

The primary goal of this systematic analysis is to determine the predictive significance of proliferative markers in surgical margins of patients with oral squamous cell carcinoma (OSCC). A thorough literature search was done on databases like MEDLINE/Pub-Med, Cochrane and Scopus libraries for similar studies until December 2022. All the relevant original research studies (retrospective and prospective) published in the literature assessing the predictive value of proliferative markers in surgical margins in OSCC were included. Seventeen studies with 1159 patients were included. The research included here used p53, p44/p42, proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR), Ki-67, Bcl2, Nibrin, AgNORs, Cyclin B1, Cornulin, ISG 15antibodies, MCM3 in OSCC. Four studies were done on oral premalignant lesions and OSCC. Among these studies, Ki-67 was the most accurate, followed by p53 (75%) and AgNORs, while PCNA had the least accuracy. To minimize the risk of bias panel of antibodies was suggested in most studies. For interobserver variability, analysis of variance and Chi-square test were used in most studies. The chance of recurrence rate was calculated using a log-rank test and a Kaplan–Meier curve. The significance of proliferative markers in surgical margins of OSCC has been emphasized in the present review. Future research should focus on selecting antibodies, preferably a panel, with a large sample size and extended follow-up.

Keywords: AgNORs, Ki-67, p53, proliferative markers, squamous cell carcinoma

Address for correspondence: Dr. Madhuri Gawande, Department of Oral Pathology and Microbiology, Sharad Pawar Dental College and Hospital, DMIHER, Sawangi (M), Wardha – 442 001, Maharashtra, India.
E-mail: madhurigawande68@gmail.com

Submitted: 31-Aug-2023, **Revised:** 05-Sep-2023, **Accepted:** 09-Oct-2023, **Published:** 20-Dec-2023

INTRODUCTION

Oral carcinoma, constituting more than 90% of cancers arising from the oral cavity, is the most common malignancy worldwide in incidence and mortality, and also in developing countries, including India. Early detection, appropriate treatment planning and target drug therapy are critical to better prognosis and high survival rate of patients. The result and prognosis concerning treatment, cure and survival of the patient have been poor due to treatment resistance and recurrence of the disease despite

Access this article online

Quick Response Code:

Website: https://journals.lww.com/JPAT/

DOI: 10.4103/jomfp.jomfp_390_23

How to cite this article: Gawande M, Khan S, Sharma N, Hande A, Patil S. Role of proliferative markers in assessing recurrences in surgical margins of oral squamous cell carcinoma: A systematic review. J Oral Maxillofac Pathol 2023;27:735-43.

the significant advancements made over the past few years in its diagnosis, prevention and treatment. A local recurrence or metastasis affects more than 50% of patients ultimately, usually within the first two years after therapy is finished.[1]

The prognosis and treatment success are significantly influenced by the tumour size, stage, grade, location, depth of invasion, lympho-vascular dissemination and performance. Response to treatment differs from person to person, even when the tumour is in the same stage. Tumour biology variations, such as changes in cell shape or genetic phenotype, may be to blame for this. As a result, managing oral squamous cell carcinoma (OSCC) is significantly threatened by the heterogeneity of tumour cells. Recent developments in molecular biology and a better understanding of the pathophysiology of OSCC have opened up a wide range of new study directions.[2] Numerous studies have examined the relationship between these markers' expression, treatment response and survival. Still, relatively few have linked it to tumour recurrence,[3] a severe issue in OSCC. Other cancers like breast, lung, colorectal, bladder and gastrointestinal tract have received the most research attention. There has been a continuing quest for new prognostic, proliferative and predictive markers to understand the tumour behaviour, treatment outcome and tendency to recur due to the most dreaded malignancy's aggressiveness and high mortality rate. By classifying patients based on risk variables found, searching for new markers is a developing strategy to individualize and facilitate treatment planning, allowing high-risk patients to be maintained under active surveillance to evaluate tumour relapse.

Proliferative immunohistochemistry (IHC) markers have not yet been thoroughly investigated in studies of OSCC cases to determine their prognostic value. The recommended Reporting for Systematic Reviews and Metanalysis was used for this study.

METHODS

Eligibility criteria

The following criteria were used to select the studies:

Study design: Prospective and retrospective original research publications published in the literature were involved from the start through December 2022. Case studies, animal experiments, reviews, conference papers, abstracts, unpublished data and articles produced by the same author but using duplicate data were all excluded.

Intervention: IHC tests on frozen sections were the only research. The studies did not include other approaches, such as real-time polymerase chain reaction (RT-PCR), DNA methylation and gene analysis.

Participants: Patients diagnosed with OSCC were included.

Outcome

Prognostic role of proliferative IHC markers to comprehend the molecular nature of the mechanism of recurrence in frozen sections of OSCC and improved quality of life in high-risk patients to assess disease relapse.

Search strategy

A voluminous search of the literature was carried out in 'MEDLINE'/'PubMed', 'Scopus' and 'Cochrane Library', 'Google Scholar' for germane articles under the Mesh terms 'proliferative markers for Recurrence in OSCC', 'frozen sections', other relevant publications were identified from the citation lists and review articles. Any unrelated publications were ruled out by thorough scanning of titles and abstracts of each recognized study.

Data extraction

Two authors independently made an extraction sheet to omit unrelated articles and publications that met the inclusion and exclusion criteria were assessed. A third author settled any differences of opinion. The author, year of study, country in which the study has been conducted, period of recruitment, number of patients, age of patients, involved site, specimen analysed, method of detection, source of antibody, cut-off value and follow-up time were all collected independently by two authors from relevant studies.

RESULTS

Search results and outcome

The initial search resulted in a total of 56 articles. Out of these, 39 articles were found irrelevant, so they were excluded. These included methods like flow cytometry, DNA cytometry, *in situ* hybridization, PCR, etc., Some of these articles were reviews, serum-based studies, etc., Finally, 17 articles that fulfilled the present review's conditions were included. [Figure 1]

General characteristics of eligible studies

The total number of patients was 1159. Twelve studies were Asian, whereas five were non-Asian. Fifteen studies included cases of OSCC from different regions, whereas two included cases of OSCC and oral leukoplakia. All the studies used cases already analysed by hematoxylin and

eosin stain, and IHC was used as a detection method. None of them had undergone chemotherapy and radiotherapy. Different follow-up period was followed in all.

Parameters related to immuno-histochemical markers of included studies

To assess the recurrence in OSCC, sundry cell proliferative markers are used alone or in amalgamation [Table 1]. Included studies used p53 in four studies[4-7] in combination with p44/p42, proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR) in OSCC[1] while with Ki-67, Bcl2 in oral leukoplakia and OSCC.[2] Cyclin B1 was used in one study,[3] AgNORs in two studies in OSCC[3,4] and one study in oral leukoplakia five and OSCC, Nibrin in OSCC,[8] Ki67 was used in eight studies along with AgNORs,[9-13] cornulin, ISG15 in OSCC,[14,15] MCMC-2[16] and three studies in oral leukoplakia and OSCC p53 from DAKO was used in dilution, 1:50 in one study along with PCNA (clone PC10) in dilution, 1:5000, phospho-p44/p42 mitogen-activated protein kinase (ERK1/ERK2; Cell-Signaling, Technology R; dilution, 1:100, EGFR Ab-10, clone 111.6; Thermo Scientific; dilution, 1:100). All gave brown staining to the nucleus of epithelial cells.[1] In another study, p53 was used with Ki-67 as p53 (BP-53-12, PM 101-6ML), and Ki-67 (GM 001-PM 096-6ML) from Gene Pulse Scientific[2]. p53 and Ki-67 were both ready to use antibodies procured from BioGenex while Bcl2 was also ready to use from Biocare Medical.[3] The epithelial cell nuclei with a distinct brown colour were considered positive for p53 and Ki-67 expression. For Bcl2, cytoplasmic light brown staining in epithelial cells was regarded positive, while lymphocyte staining served as an internal control. In another study, p53 from DAKO clone DO-7[4] was used alone. It resulted in brown-stained nuclei and AgNORs were used depending upon the protocol described by Ploton and Ki-67 from Dako Cytomation in dilution of 1:25. Both resulted in brown-stained nuclei[5]; in two studies, AgNORs were freshly prepared according to the protocol described by Ploton *et al*.[18] and the AgNOR dots were identified as black dots in brown-stained nuclei.[6,7] In one study Nibrin from Santa Cruz Biotechnology was used in a dilution of 1:100, with positive controls as formalin-fixed intense staining for a given marker. It was evaluated with the nuclear location of the immuno-reactions.[7] Ki-67 was used in various studies alone or combined with other markers. In one study, Ki-67 from Novacastra was used in a dilution of 1:100 in combination with PCNA from DAKO in a dilution of 1:2000. Cyclin B1 from Novocastra in dilution of 1:40. Positive nuclear stain was seen in Ki-67 and PCNA and cytoplasmic stain for Cyclin B1.[17] In another study it was used with AgNORs,[5] used with the combination of Ki-67 and p53,[2] and in another Ki-67, p53 and Bcl2 were used.[3] Ki-67 was used alone in five studies.[9-13] Out of which, four studies used Ki-67 from DAKO,[9-11,13] and one study was from Leica Biosynthesis,[12] which used positive control as ameloblastoma and Tris buffer saline as a negative control; all showed brown discolouration of nuclei of epithelium. In one study, Ki-87 was used with MCM3, both from DAKO, in dilution of 1:150 and 1:100, respectively. For both, breast carcinoma was used as a positive control. Immunoreactivity was mainly in the nuclear region.[16] In another study, Ki-67 antigen in dilution 1:300 from DAKO, Cornulin-antibody in dilution 1:300 from Proteintech and ISG15 antibody in dilution 1:100 from Sigma-Aldrich were used.[15] All showed positive brown nuclear staining. In another study, MCM2 from Biogenex and Ki-67 also from Biogenex were used. Both showed positive nuclear staining.

Immunohistochemical studies: Potential markers

Archived blocks and paraffin-embedded sections of surgical margins of OSCC fixed in formalin were taken in all the studies [Table 2]. In a study, the association between expression of p53, EGFR, PCNA, p44/42 and clinical staging along with its recurrence was studied in 48 patients of OSCC, and no significant correlation was found.[1] Fifty recurrent and non-recurrent OSCC cases were studied to find the correlation between the immuno-expression of p53 and Ki-67 and the prognosis of OSCC. They found a positive correlation between clinicopathological parameters and the labelling index of these two markers.[2] In another study, 30 cases of oral leukoplakia and OSCC were taken, and immuno-expression of p53, Ki-67 and Bcl2 were assessed and correlated with their clinicopathological parameters. They noted significantly high p53 and Ki-67 expression in OSCC compared to oral leukoplakia and can be used as prognostic markers.[3] In another study, 30 cases of OSCC were taken.

Immuno-expression of p53 was studied in the tumour-invasive front; the overexpression of p53 showed that it could be used as a prognostic marker in OSCC.[4] The combination of AgNORs and Ki-67 was studied in 40 cases of OSCC.[5] A statistically non-significant correlation was found between the immuno-expression of Ki-67 and AgNORs and tumour-invasive front, regional metastasis and patient prognosis. The AgNORs count was done in 15 cases of oral leukoplakia and 20 cases of OSCC[6]; they found it to be higher in OSCC cases and thus concluded that it could be used as a prognostic marker in OSCC. Through digital image analysis, 43 cases of tongue carcinoma and carcinoma of the floor of the mouth were analysed to examine the AgNORs area and nucleus. They found a statistically significant correlation between AgNORs expression and vascular invasion, lymph

Table 1: Parameters of immunohistochemical markers used in included studies

Antibodies	Source	Dilution	Expression
p53, EGFR, PCNA, p44/4	DAKO, Thermo Scientific, Cell Signaling Technology	p53-1:50, EGFR-1:100, PCNA-1:5000, p-14/4-1:100	Brown nucleus staining epithelial cells
p53, Ki-67	Gene Pulse Scientific	p53-PM 101-6ML, Ki-67-PM 096-6ML	
p53, Ki-67 Bcl2	BioGenex, Biocare Medical	ND	
p53	DAKO	ND	
AgNORs, Ki-67	DAKO AgNORs by Ploton *et al*.	Ki-67-1:25,	
AgNORs	AgNORs by Ploton *et al*.	ND	
Fibrin	Santa Cruz Biotechnology	1:100	
PCNA, Ki-67, Cyclin B1	DAKO, Novocastra	PCNA-1:2000, Ki67-1:100, Cyclon B1-1:40	
Ki-67	DAKO, Leica Biosystemss	ND	
Ki-67 and MCM3	DAKO	Ki-67-1:150, MCM3-1:100	
Ki-67, Cornulin and ISG15	DAKO, Proteintech, Sigma-Aldrich	Ki67-1:300, Cornulin-1:300, ISG15-1:100	
MCM@, Ki-67	Biogenex, DAKO	ND	

Table 2: Summary of the characteristics of included studies

Author	Year	Country	Recruitment period	Sample size	Age range (years)	Tumour type	Specimen analysed	Detection method	Follow-up
Świątkowski[1]	2017	Poland	2002–2006	48	40–48	OSCC	Tissue	IHC	No follow-up
Babu *et al*.[2]	2020	India	2018–2019	50	20–40	OSCC	Tissue	IHC	3 years
Bhattacharya *et al*.[3]	2017	India	Jun 2016–Sep 2016	30	40–70	Oral leukoplakia and OSCC	Tissue	IHC	3 years
Gawande *et al*.[4]	2021	India	2020–2021	30	20–50	OSCC	Tissue	IHC	3 years
Veronica *et al*.[5]	2016	Brazil	2000–2010	109	30–60	OSCC	Tissue	IHC	2 years
Mehkri *et al*.[6]	2010	India	2009–2010	35	30–70	Oral leukoplakia and OSCC	Tissue	IHC	2 years
Teixeira *et al*.[7]	1996	Brazil	1987–1992	43	20–60	OSCC	Tissue	IHC	2 years
Dave *et al*.[8]	2015	India	2011–2013	100	30–40	OSCC	Tissue	IHC	3 years
Watanabe *et al*.[17]	2010	Brazil	1996–2000	39	30–60	OSCC	tissue	IHC and Image Pro Plus software	2–3 years
Dash *et al*.[9]	2020	India	2019–2020	100	20–50	Oral premalignant and OSCC	Tissue	IHC	2 years
Chaudhari *et al*.[10]	2018	India	July 2015–Dec 2018	100	20–60	OSCC	Tissue	IHC	2 years
Maheshwari *et al*.[11]	2018	India	2017–2018	63	30–70	Oral premalignant and OSCC	Tissue	IHC	3 years
Bhuyan *et al*.[12]	2018	India	2017–2018	27	20–60	OSCC	Tissue	IHC	2 years
Jing *et al*.[13]	2018	China	2007–2014	62	30–70	OSCC	Tissue	IHC	2 years
Lopes *et al*.[14]	2017	Brazil	2016–2017	51	20–60	OSCC	Tissue	IHC	3 years
Govindaraj *et al*.[15]	2021	Malaysia	2020–2021	34	20–60	OSCC	Tissue	IHC	2 years
Kumar *et al*.[16]	2018	India	Dec 2016–March 2018	30	30–70	OSCC	Tissue	IHC20-70	2–3 years

node metastasis and surgical margins of tumour.[7] Nibrin expression was analysed in 100 cases of carcinoma of the tongue and buccal mucosa.[8] They found a statistically significant correlation between the expression of Nibrin and poorly differentiated OSCC and thus could be used as a prognostic marker. Thirty-nine cases of OSCC were analysed to assess the immuno-expression of PCNA, Ki-67 and Cyclin B1. Higher expression of all markers was observed, and it concluded that they all could be related to the prognosis of patients.[9]

Ki-67 was used alone in five studies.[9-13] In a study, there were 45 cases of oral premalignant lesions and 45 cases of OSCC[9]; in another study, there were 100 cases of OSCC[10] and 65 cases of oral premalignant and oral cancer.[11] Similarly, 102 cases of OSCC[12] and yet another study of 298 cases of OSCC[13] were studied to assess the immuno-expression of Ki-67. They found increased expression, and a statistically significant difference was seen in increasing grades so that it can be used as a prognostic marker. In 51 cases of OSCC, the immuno-expression of Ki-67 and MCM3 were analysed.[14] Ki-67 expression was positively and statistically correlated with the survival rate of patients, whereas no such correlation with MCM3 expression was found. In another study, surgical margins of 32 cases of OSCC were evaluated for the immuno-expression of Ki-67, Cornulin and ISG15 antibodies. A negative correlation was found for Ki-67 and ISG15 antibodies.

In contrast, a low expression of Cornelia was found, which could be used as an independent prognostic marker.[15] The combination of Ki-67 and MCM2 was studied in 30 cases of OSCC. A high and statistically significant labelling index was noted for both markers in negative surgical margins. So, they can be used as novel markers for predicting recurrence in OSCC.[16]

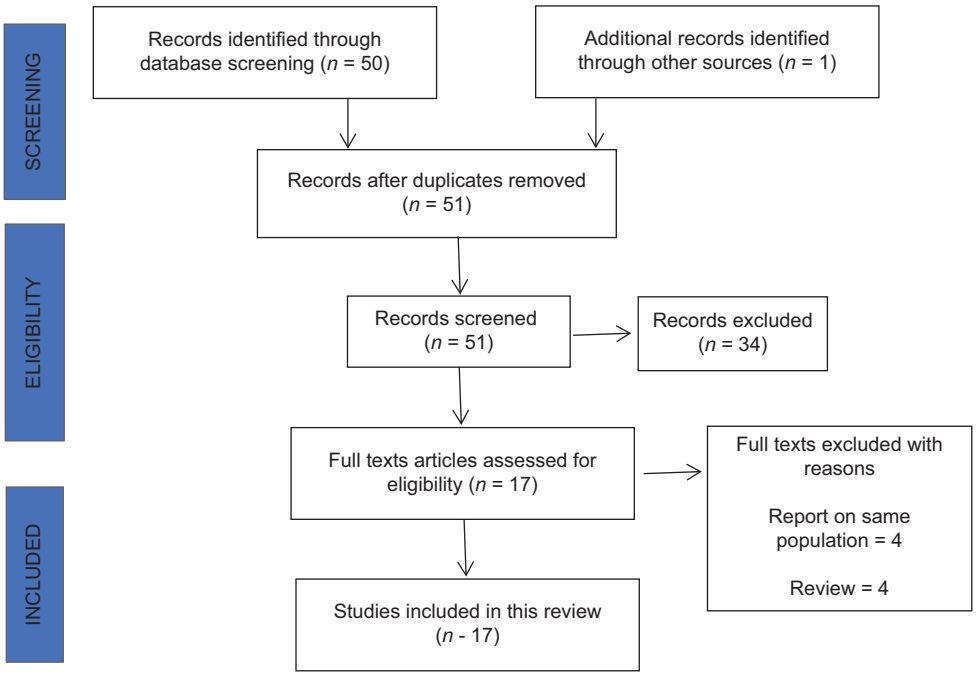

Figure 1: Flowchart of methodology according to Prisma Guidelines

Significance of potential markers

The proliferation condition of a cell or tissue with the expression of the Ki-67 marker commonly analyses the proliferation-related nuclear antigen. A gene on chromosome 10q25 encodes Ki-67. Ki-67 expression occurs in all cell cycle stages except G0 and early G1. It increases in the S phase, peaks in the G2 and M phases, and degrades rapidly after mitosis. Ki-67 expression in proliferating cells, DNA synthesis, apoptotic activity and OSCC is important and can be used to anticipate the histologic grade of differentiation and prognostic condition of the lesion. Studies have shown overexpression of Ki-67 in the supra-basal epithelium, increasing with the severity of dysplasia, and hence has been correlated with poor survival.[2,3,5,9-13,17]

The proliferation rate of cells in tissues can determine the biological behaviour of tumours and can be used as one of the markers for predicting malignancy in potentially malignant diseases. To control protein synthesis and proliferation, the nucleus is crucial. As determined by Ki-67, the percentage of S phase cells and the percentage of mitotic cells, AgNORs corresponds with the proliferation rate. AgNORs staining involves impregnating colloidal silver with particular proteins that are linked to the transcriptional activity of the nucleolar organizer regions (NORs). NORs are DNA loops that house ribosomal genes responsible for synthesizing the 18S and 28S subunits of ribosomal RNA (rRNA). To control protein synthesis and proliferation, the nucleus is crucial. As determined by Ki-67, the percentage of S phase cells and the percentage of mitotic cells, AgNORs corresponds with the proliferation rate. AgNORs staining involves impregnating colloidal silver with particular proteins that are linked to the transcriptional activity of the NORs. NORs are DNA loops that house ribosomal genes responsible for synthesizing the 18S and 28S subunits of ribosomal RNA (rRNA). For this reason, they are known as argyrophilic nucleolar proteins or AgNORs. The quantity of AgNORs per nucleus shows that it is a quantitative indicator of the proliferative activity of the cell. AgNORs serve as a marker of premalignant or malignant transformation qualitatively (based on the shape, size and distribution pattern). AgNOR levels are correlated with cell cycle progression, increasing from G0 to S-phase, and are inversely correlated with neoplastic cell proliferation.[8] A tumour population that divides quickly is more likely to contain a higher proportion of cells in the early stages of G1 before individual NORs have been associated, making it more probable that these cells will be seen in more instances. On the other hand, NORs are more prevalent in tumours with low cell growth rates. The RNA polymerase I, B23, C23 and 'AgNORs' proteins linked with these rRNA transcription sites are identified using silver staining rather than rRNA or rDNA.[17] The recent allegations that NORs are substantially more common in malignant cells than in normal, reactive or benign neoplastic cells have gained much attention.[5-7] The most frequent anomaly in different tumours is the p53 tumour suppressor gene mutation. Missense mutations, dispersed throughout the gene's core region, account for over 95%

of all mutations. Although each of these mutations turns off the biological functions of the p53 protein, their impact on p53 stability is significant. The mutant p53 protein, which takes on an abnormal form and is more stable than the wild type (half-life of several hours compared to 20 min for the wild type p53) and accumulates in cancer cells' nuclei, becomes immunologically detectable. Positive immunostaining indicates that the p53 gene and its product have been altered due to this occurrence. Neurosurgeons can benefit from intraoperative frozen section diagnosis of astrocytic tumours for many reasons: (1) to confirm the excised tissue's neoplastic character and (2) to identify grade IV anaplastic gliomas from benign neoplasms and gliotic tissue. Using Ki-67 IHC, tumour proliferation activity can be evaluated.[1-4]

A change in cell proliferation is a defining feature of tumour progression. It will be beneficial in prognosis if we can analyse it. The proliferation marker PCNA is a proliferative nuclear antigen protein. It is well known that it affects the prognosis and survival of breast and colorectal cancer. It builds up during the cell cycle's late G1 and early S phases.[14,15] Studies have shown a positive connection between OSCC histological grading and PCNA expression. PCNA expression varies between normal and dysplastic epithelium, as well as between benign and malignant lesions. Also, PCNA expression was directly correlated with the histologic grading of OSCC. Increased PCNA expression was seen in poorly differentiated OSCC, and decreased expression was seen in well-differentiated OSCC. The EGFR is a membrane-bound tyrosine receptor that is activated in tumour cells of epithelial origin. Thoreaulites undergo growth, proliferation, apoptosis, differentiation, migration and protein production. EGFA expression has been linked to cancer progression, including metastasis.[1,2]

A family of serine/threonine-specific protein kinases known as mitogen-activated protein kinases were previously described as proteins triggered by cell stimulation or growth stimuli. It can be divided into p38 kinases, C-Jun N-terminal stress-activated protein kinases, and extracellular signal-regulated kinases (ERK). Two homologous versions of the ERK kinase exist ERK1 (P44) and ERK2 (p42). The expression of and the clinical cancer stage did not correspond.[1]

The membrane-bound tyrosine kinase receptor EGFR is activated in epithelial tumour cells. It controls protein secretion, migration, differentiation, apoptosis and other cellular processes. Its activation may contribute to cancer metastasis, a cancer progression during mitosis. Subcellular structures undergo phosphorylation due to the activation of Cyclin B1 complex. It forms a complex known as MPF on combining with another protein CDK. It reaches peak concentration in mitosis and thus helps in the proliferation of malignant cells.[2]

For recognition and the repair of double-strand breakages, an amino acid protein called Nibrin is involved. The Nibrin-containing protein complex [Mre11-Rad50-Nbs1(MRN) complex] binds to the margins of the DNA double-strand break and remains there until it is repaired. It also plays a role in telomere preservation, meiotic recombination and mitotic V(D)J rearrangements in T and B lymphocytes. It is overexpressed in reproducing cells.[1]

As an anti-apoptotic protein, the location of Bcl2 is in cellular organelles like membranes of mitochondria, nucleus and endoplasmic reticulum; many researchers have postulated the correlation between the expression of Bcl2 with cancer initiation, growth and progression. Its overexpression could be an indicator of poor prognosis of oral cancer.[3]

Mini-chromosome maintenance proteins (MCM) are thought to play a role in the early stages of eukaryotic genome replication. Its family includes six members, i.e. MCM2 to MCM7. All play an equal part in the replication of DNA. Many studies have proved that when the cell enters the quiescent state, there is a disappearance of expression of MCM3 proteins or MCM2 proteins, which could be an early indicator to differentiate between proliferating and resting cells. And they could be used as novel biomarkers in predicting recurrences in surgical margins of OSCC.[14]

Squamous epithelial heat shock protein-53, known as the Cornulin gene, functions as a stress-responsive factor. Many molecular studies conducted in the past have shown that its expression is predicted as a survival factor in humans; its upregulation is seen in many studies, like in psoriasis of the skin or in the buccal mucosa of smokers. Thus, its upregulation is correlated with healthy epithelial tissues, but under-expression is associated with cancerous lesions, as seen in oesophageal squamous cell carcinoma and OSCC. Hence could be used as an early prognostic indicator of recurrence.[15]

ISG15 is an interferon-regulated gene mainly induced by various microbial and cell stress stimuli and functions as a tumour suppression factor. Many studies conducted in the past have proved that the overexpression of ISG15

was seen in surgical margins of OSCC than in adjacent normal tissue. This could be used as a potential biomarker of cancer progression in OSCC and as a prognostic marker in surgical margins of OSCC which could assess recurrences.[15]

DISCUSSION

In routine hematoxylin and eosin examination of surgical margins of head and neck cancers, the regular use of IHC markers has yet to be established. The prime importance of selecting the standard treatment protocols to prevent recurrence and improve the survival rate of OSCC patients is the accuracy and reliability of the markers used. The primary purpose of the present systematic review is to amalgamate the current data on the role of proliferative markers in assessing recurrence in negative surgical margins of OSCC. A total of 17 articles fulfilled our inclusion criteria. Flow cytometry, DNA cytometry, animal studies, review articles, RT-PCR, genetic analysis and unpublished data were excluded from the present study.

As few studies were included in the present systematic review, limited data could be collected with further need for randomized controlled trials with long-term follow-up. These studies should focus mainly on the evaluation of new markers or more appropriately combination of markers in OSCC patients.

Different IHC markers can be used in negative surgical margins of OSCC. The studies used p53, p44/p42, PCNA, EGFR, PCNA, Ki-67, Cyclin B1, AgNORs, Nibrin, MCM3, MCM2, Cornulin, ISG15 antibodies in OSCC while Bcl2, Ki-67, AgNORs in oral leukoplakia and OSCC cases. Different studies showed differences in results which could be because of methodological issues, varied sample sizes and different follow-up times.

In a study, p53, EGFR, PCNA and p44/42 in the tumour mass and the clinical stage of disease, only p53 showed a significant relation with the staging of the tumour. It could be combined with other markers to find the severity of the disease.[1] In another study, the combination of p53 and Ki-67 was studied in recurrent and non-recurrent OSCC.[2] The labelling index of both markers was found to be higher in recurrent OSCC. Overexpression of both was correlated with survival and prognosis. Another study analysed the combination of p53, Ki-67 and Bcl2 in oral leukoplakia patients and OSCC patients.[3,4] A highly significant expression of p53 and Ki-67 was seen in moderately to poorly differentiated OSCC.

In contrast, an expression of Bcl2 showed no significant correlation with both groups and other factors.[2,3] Thus they concluded that the expression of p53 and Ki-67 could be correlated with poor prognosis and recurrence of high-risk cases. Another study analysed the expression of p53 with a tumour-invasive front and showed overexpression with increasing grade with poor prognosis and high recurrent rate.[4]

The combination of Ki-67 and AgNORs was used in the invasive front of OSCC.[5] No statistically significant difference was seen between the expression of Ki-67 and AgNORs, regional metastasis and the patient's prognosis. They are not prognostic markers at the invasive front of OSCC. AgNORs count was done in oral leukoplakia and OSCC cases.[6] They were found higher in OSCC and with increasing grades of oral leukoplakia could be used as a prognostic marker. The expression of AgNORs was seen in OSCC cases of tongue and floor of mouth.[7] They found an increase in AgNOR count in higher grades and could be used as a prognostic marker in high-risk patients. In a study, Nibrin was used in OSCC cases; it showed a positive correlation with moderately and poorly differentiated SCC cases and a statistically significant correlation with disease reoccurrence in early stages so that it can be used as a prognostic marker.[8]

Ki-67 was used in many studies, either alone or with other markers. In two studies, Ki-67 was used in oral leukoplakia and OSCC cases.[9,11] Both studies showed an increase in the expression of Ki-67 with disease progression. Also, it could be used as an early prognostic marker to check recurrences. In the other three studies, Ki-67 was used alone in OSCC cases.[11-13] They all showed increased expression with an increase in the grade of tumor. They showed a positive correlation between lymph node metastasis and poor prognosis, a very efficient tool for predicting recurrence. In another study, PCNA, Cyclin B1 and Ki-67 were used in OSCC cases in tumour-invasive front. They found a positive correlation with Ki-67 and Cyclin B1[2] expression, whereas a statistically non-significant relation with PCNA expression. They concluded that a combination of Ki-67 and Cyclin B1 is effective in predicting the degree of proliferation of the tumour and prognosis. The combination of Ki-67 and MCM3 was used in another study in OSCC patients.[14] They found a positive correlation between the expression of Ki-67 with tumour size, stage, metastasis and recurrence. Ki-67, Cornulin and ISG15 antibodies were studied in the surgical margins of OSCC cases.[15] They found a statistically non-significant correlation between the expression of Ki-67 and ISG15 antibodies with age, sex and local relapse.

In contrast, the expression of Cornulin was reduced in these cases, which was positively correlated with relapse, so it could be used as an independent marker to show relapse in surgical margins of OSCC. In another study, a combination of Ki-67 and MCM3[14] was studied in the surgical margins of OSCC. They showed statistically significant results with both markers. So, the expression of Ki-67 was correlated with disease and nodal metastasis and MCM2 expression with tumour size and staging, so MCM2[16] could be a novel biomarker in predicting recurrence and survival in negative surgical margins of OSCC.

Quantitative analysis of the IHC analysis was used in all the studies. Labelling index of proliferative markers was calculated by multiplying the number of positive cells by 100 and dividing it by the total number of tumour cells observed. Staining intensity ('SI') and the proportion of positive cells are multiplied to produce the intensity reactivity score. In a few studies primarily focused on cellular localization, SI and percentage of positive cells, semi-quantitative analysis was also recommended. In one investigation, the labelling index was examined using image analysis tools.[2]

Mann–Whitney, Kruskal–Wallis, Spearman's rank correlation coefficient test and Fisher's exact tests were used to compare the positivity of markers and clinicopathological parameters among groups. To investigate the connection between molecular markers and clinical characteristics, contingency tables and the two-test were used. Univariate and multivariate Cox regression analyses were utilized to evaluate the combination of indicators for their significance in the patients' recurrence-free survival. Interobserver variability was assessed using the Kappa method. To analyse the likelihood of recurrence rate, we used the log-rank test and a Kaplan–Meier curve.[1-4,6,8,9,11-14]

In a systematic review, certain limitations will always be part. These arise due to differences in their inclusion and exclusion criteria; only some studies included lymph node metastasis which was excluded in two studies. Included studies had all OSCC cases except four studies which included both premalignant lesions and OSCC cases. The main difference in studies was its sample size which affected the results resulting in low to critical bias in confounding factors. The vast diversity in biomarkers used and different follow-ups also created a significant limitation. All these create difficulty for a standard prognostic biomarker in cancer patients. Thus, more extensive studies in future are needed with elaborative data on IHC markers, along with a large sample size and longer follow-up time will be much more beneficial.

CONCLUSION

The present systematic review reflects that the immunostaining of proliferative markers in negative surgical margins can significantly contribute to head and neck cancers. The main focus in future should be adequate sample size, longer follow-up and selection of appropriate prognostic biomarkers, preferably a panel and qualitative as well as quantitative analysis. This will aid the development of a viable predictive marker for head and neck cancer surgical margins.

Acknowledgement

The authors are thankful to all the participants for their contribution and support in this study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Świątkowski W, Rahnama M, Tomaszewski T, Bigas M, Świątkowska A, Łobacz M, *et al*. Association between clinical stage of oral cancer and expression of immunohistochemical markers. Pol Przegl Chir 2017;89:17-22.
2. Babu B, Hallikeri K, Kumar K. Immunoexpression of p53 and ki-67 correlated with clinicopathological parameters in predicting recurrence of oral squamous cell carcinoma. Asian J Med Sci 2020;1:1-8.
3. Bhattacharya I, Dawson L, Sharma S. Prognostic significance of *p53*, Ki-67 and *Bcl-2* in leukoplakia and squamous cell carcinoma of the oral cavity. Natl J Lab Med 2017;6:16-21.
4. Gawande M, Chaudhary M, Sharma P, Hande A, Patil S, Sonone A. Expression of p53 at invasive front of oral squamous cell carcinoma and negative histopathological surgical margins to establish correlation at 3-year survival. J Oral Maxillofac Pathol 2020;24:582.
5. Veronica A, Cortegoso BI, Laureano NK, Dutra A, Danilevicz CK, Magnusson AS. Cell proliferation markers at the invasive tumor front of oral squamous cell carcinoma: Comparative analysis in relation to clinicopathological parameters of patients. J Appl Oral Sci 2017;25:318-23.
6. Mehkri S, Iyengar AR, Nagesh KS, Bharati MB. Analysis of cell proliferation rate in oral leukoplakia and oral squamous cell carcinoma. J Clin Exp Dent 2010;2:173-7.
7. Teixeira, G, Antonangelo L, Kowalski L, Saldiva P, Ferraz A, Silva Filho G, *et al*. Argyrophilic nucleolar organizer regions staining is useful in predicting recurrence and free interval in oral tongue and floor of mouth squamous cell carcinoma. Am J Surg 1996;172:664-8.
8. Dave JH, Vora HH, Trivedi Trupti, Ghosh N. Nibrin expression in oral squamous cell carcinoma: Association with clinicopathological parameters. J Cancer Metastasis Treat 2016;2:436-42.
9. Dash KC, Mahapatra N, Bhuyan L, Panda A, Behura SS, Mishra P. An immunohistochemical study showing Ki-67 as an analytical marker in oral malignant and premalignant lesions. J Pharm Bioall Sci 2020;12:S274-8.
10. Chaudhari M, Shukla S, Tamhane A, *et al*. Histopathological grading

of oral squamous cell carcinoma and its correlation with ki-67 -a proliferative marker. J Evid Based Med Healthc 2018;5:1690-5.
11. Maheshwari V, Sharma SC, Narula V, Verma S, Jain A, Alam K. Prognostic and predictive impact of Ki-67 in premalignant and malignant squamous cell lesions of oral cavity. Int J Head Neck Surg 2013;4:61-5.
12. Bhuyan L, Sarangi S, Das BK, Das SN, Nayak S. Proliferative index in invasive tumor front of oral squamous cell carcinoma: A potential prognostic indicator. J Contemp Dent Pract 2018;19:170-6.
13. Jing Y, Zhou Q, Zhu H, Zhang Y, Song Y, Zhang X, *et al*. Ki-67 is an independent prognostic marker for the recurrence and relapse of oral squamous cell carcinoma. Oncology letters 2019;17:974-80.
14. Lope VK, Jesus AS, Souza LL, Miyahar HA, Guimarães DM, Pontes HA. Ki-67 protein predicts survival in oral squamous carcinoma cells: An immunohistochemical study. Braz Oral Res 2017;31:66-72.
15. Govindaraj PK, Kallarakkal TG, Mohd Zain R, Tilakaratne WM, Lew HL. Expression of Ki-67, Cornulin and ISG15 in non-involved mucosal surgical margins as predictive markers for relapse in oral squamous cell carcinoma (OSCC). PLoS One 2021;16:2-12.
16. Kumar K, Hallikeria K, Anehosurb VS, Kumar Nb, Desai AK. MCM 2 a novel marker in predicting recurrence and prognosis at negative surgical margins of oral squamous cell carcinoma. J Oral Maxillofacial Surg Med Pathol 2019;1:1-6.
17. Watanabe S, Watanabe S, Oton A, Alenear R, Oliveira J. Analysis of cell proliferation and pattern of invasion in oral squamous cell carcinoma. J Oral Sci 2010;52:417-24.
18. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ. Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J 1986;18:5-14.