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Role of Immunohistochemical Markers in Surgical Margins of Patients with Head and Neck Carcinoma – A Systematic Review

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ABSTRACT

Objective(s): The aim and objective of this study was to systematically evaluate the prognostic role of immunohistochemical markers in surgical margins of patients with head and neck squamous cell carcinoma (HNSCC).

Materials and Methods: MEDLINE/PubMed, Scopus and Cochrane library were searched for relative studies until December 2018. Retrospective and prospective original research studies published in English language assessing the prognostic value of immunohistochemical markers and disease-free survival in HNSCC and oral squamous cell carcinoma (OSCC) were included.

Results: A total of eight studies were included comprising of 269 cases. The studies included here used eukaryotic initiation transcription factor 4E (eIF4E) in HNSCC patients; Matrix metalloproteinase (MMP), MMP2, MMP3, MMP9, Dentin Sialo-Phosphoprotein (DSPP), Bone Sialoprotein (BSP), Osteopontin (OPN), Beta-2-adrenergic receptors and E-cadherin in OSCC patients and p53 in HNSCC and OSCC. Among all the markers studied MMP9 had the highest accuracy at 80% followed by p53 (75%), DSPP (70%) and OPN (70%) while eIF4E (33.3%) had least accuracy. A study suggested that E-cadherin is the preferred marker over MMP9. Almost all the studies used Fisher's exact and Fisher-Freeman-Halton significance test. Only one study was at low risk of bias, three studies were at moderate risk of bias, three studies had serious risk of bias and in one study bias could not be calculated due to inadequate information.

Conclusions: The study shows that immunohistochemical markers can significantly contribute to the field of head and neck carcinomas. Future efforts should concentrate on improving the antibody selection and its performance in the patients.

Key Words: E-cadherin, eIF4E, Matrix Metalloproteinase, Osteopontin, p53, Squamous cell carcinoma

INTRODUCTION

Head and neck cancer are the eighth most common cancer worldwide with majority being head and neck squamous cell carcinoma (HNSCC). HNSCC arise in the epithelial linings of the oral cavity, sino-nasal tract, pharynx, larynx and paranasal sinuses [1]. HNSCC is increasing rapidly since past decades and oral squamous cell carcinoma (OSCC) is the

commonest tumor of head and neck region [2]. Despite various new advances in diagnosis and management, the long-term survival in HNSCC patients has not improved considerably.

The primary mode of treatment till date is surgery which may be followed by chemotherapy and radiotherapy depending upon the individual case [3]. To appropriately demarcate

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the exact margins is a dilemma for any surgeon. The presence of carcinoma in or close to the margin is an imperative prognosticator which could influence the local relapse of the patients [4]. The histopathologically tumor positive surgical margins are prognostic indicator for tumor relapse or distant metastasis. But relapse often occurs in cases with clear margins as well. This is in support with the hypothesis by Slaughter et al. [5] that residual or altered field in proximity to the primary tumor might be the principal cause of local recurrence and treatment failure.

Immunohistochemical assessment of clear surgical margins could represent a more sensitive approach to detect the minimal residual cancer in them as genetic alteration precedes the phenotypic changes of the epithelium. Few studies have been done focusing on identification of immunohistochemical markers which may provide prognostic information and persuade the clinical outcome of HNSCC patients. The assessment of margins using immunohistochemistry (IHC) can be utilized by surgeons to provide better treatment to the HNSCC patients.

To the best of our knowledge, no study has systematically evaluated the prognostic role of immunohistochemical markers in histopathologically negative surgical margins of patients with HNSCC and specifically OSCC. This research followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines [6].

METHODS

Eligibility Criteria

The studies were chosen based upon the following criteria:

Study design: Retrospective and prospective original research studies published in English language were included from the date of inception to December 2018. Case series, animal studies, review papers, conference papers, abstracts and unpublished data, article published by same author with duplication of data were excluded.

Intervention: Only immunohistochemistry (IHC) studies were included. Studies that used other methods like RT-PCR, DNA methylation, gene analysis were excluded.

Participants: Patients diagnosed with SCC and treated with surgery having histopathologically negative (without dysplasia) surgical margins were included. Sites other than Head and neck region were excluded.

Outcome: Prognostic value of immunohistochemical markers and disease-free survival in HNSCC and OSCC.

Search Strategy

We conducted a systematic literature search online in MEDLINE/PubMed, Scopus and Cochrane library to identify pertinent articles. Following search terms and combinations

were used as follows: (HNSCC) or (OSCC) and (Surgical margins). Citation lists from all retrieved studies were used to identify other relevant publications. Review articles were also scanned to recognize other related studies. Title and abstract of each recognized study were scanned to rule out any unrelated publications.

Data Extraction

Based on the title and/or abstract, two authors independently excluded irrelevant articles. After that, the remaining full text publications was assessed by two authors to review information according to the inclusion and exclusion criteria, and disagreement was resolved by third author. Two independent authors extracted all data of eligible publications. The following data were extracted from the received papers: author, year, country, recruitment period, number of patients, age range, tumor site, specimen analyzed, detection method, antibody source, cutoff value, follow-up time. Subsequently, the following information was extracted to clarify the association between expression of molecular markers and clinicopathological parameters including the gender, histopathological grade, size of tumor, lymph node metastasis, TNM stage based on the American Joint Committee on Cancer (AJCC), treatment, recurrence and disease free survival.

Quality Assessment

The Cochrane ROBINS-I (Risk of Bias in Non-randomized Studies-of Interventions) [7] tool was used for the quality assessment of the included studies. The quality assessment was carried out by two authors and disagreements were resolved by a third author. ROBINS-I tool include the use of signaling questions to guide the risk of bias judgments within seven bias domains. The studies were assessed based on the following domains: confounding; selection of participants; classification of interventions; deviations from intended intervention; missing data; measurement of outcomes; selection of the reported results. An overall estimation risk of bias was calculated at the end. (Supplementary table)

Results

Search Results and Outcome

Figure 1 shows the study search process. A total of ninety-seven articles were yielded in the initial search strategy. After screening of the titles and abstracts, sixty-six articles were excluded as they were found irrelevant. Among the remaining, twenty full text articles were screened and thirteen were excluded for the following reasons- i) Two were review articles, ii) One article was from same author and had data duplication, iii) Ten articles had interventions other than IHC. One relevant full text article was found from the screened citation lists. Eventually, eight full text publications were included in the present systematic review which met the criteria for qualitative synthesis.

General Characteristics of eligible studies

The main characteristics of the eligible studies are summarized in table 1. The total number of patients was 269, three articles were from Asian [1,11,12], and five were from non-Asian ethnicity [8,9,10,13,14]. Four studies composed of OSCC [10,12-14], one study composed of OSCC with Oropharynx SCC [8] and three composed of HNSCC [1,9,11] patients. All analyzed histopathologically negative surgical margin specimens and immunohistochemistry was used as detection method. One study used western blot as primary detection method and IHC was used for confirmation [9]. Follow-up period was varied in all the studies.

Immunohistochemical markers related parameters of included studies

There are varied IHC markers which could be used for detection of residual cancer in surgical margins of HNSCC patients. The studies included here used eIF4E in HNSCC patients; MMP2, MMP3, MMP9, DSPP, BSP, OPN, Beta-2-adrenergic receptors and E-cadherin in OSCC patients while p53 in both HNSCC and OSCC patients. p53 antibody was used in three studies, in one study the dilution is not mentioned and was sourced from oncogene science [8] and in other two studies it was used at 1:200 dilution sourced from dako and pre-diluted from ventana laboratory and it stained nuclei brown [10,11]. Three studies used eIF4E antibody at dilution of 1:500, the antibody expressed as reddish-brown peri-nuclear staining and the positive control used were Zenker's diverticulum and Breast carcinoma tissue [1,9,11]. MMP2, MMP3, DSPP and BSP were used in one study at 1:100 dilutions and expressed as reddish-brown stain [10]. MMP9 was used in two studies at 1:100 and 1:20 dilution sourced from SantaCruz biotechnology and novocastra respectively, it stained cytoplasm brown [10,12]. Osteopontin (OPN) was used in two studies at 1:100 and 1:250 dilutions sourced from SantaCruz biotechnology and Abcam respectively, it gave dark brown membranous and cytoplasmic staining [10,12]. Human colon carcinoma tissue was used as positive control for OPN in one study [13]. E-cadherin was used in one study at a dilution of 1:50 and it stained nuclei and cell membrane brown [13]. One study used Beta-2 adrenergic receptor at 1:50 dilution which stained plasma membrane and cytoplasm brown [14]. Vascular smooth muscle was taken as internal control for the antibody.

Immunohistochemical studies: Potential markers

A list of studies on immunohistochemical markers with potential to identify tissue at risk of local relapse is depicted in Table 3. All the studies that used formalin-fixed paraffin embedded margins taken from adjacent to tumor were included. p53 was used to examine twenty-four tumor free surgical margins in two different studies out of which four-

teen and thirteen margins expressed positively for antibody and ten and three patients had recurrence respectively [9,11]. Twenty-four surgical margins were evaluated using eIF4E antibody out of which twenty-one margins exhibited positive expression and six patients showed recurrence [11]. Twenty resection margins were evaluated using MMP2, MMP3 and MMP9 out of which twelve demonstrated positive expressions for MMP2, thirteen expressed for MMP3 and six margins expressed for MMP9 antibody and nine patients had recurrence [10]. MMP9 and E-cadherin were used to evaluate fifty-eight tumor free surgical margins, but the recurrence data had not been described in the study [12]. OPN was used to examine twenty negative resection margins out of which eleven expressed positively for the antibody and nine patients suffered recurrence [10]. Twenty negative surgical margins were evaluated for BSP amongst which thirteen were positive for antibody and nine patients had recurrence [10]. Beta-2-adrenergic receptor was used to evaluate sixty-two tumor free surgical margins amongst that fifty confirmed positive expression for the antibody and twenty-six patients had recurrence [14].

Immunohistochemical studies: case-control marker studies

Table 4 shows four studies with ten comparisons that have addressed the possible clinical efficacy of IHC markers for predicting local recurrence. Only studies that used a case-control approach, measuring the marker performance in a group with (cases) and without (controls) the development of local recurrence as end point have been included. Margin samples, deep or mucosal formalin fixed paraffin embedded were the tissue source of these studies and were always reported to be tumor free on routine histopathological examination. The studies show that this kind of research is in a 'learning' phase at current stage, since most markers have not been validated in an independent set of patients. The number of patients was twenty in two studies and was twenty-four in two studies [8,10,11,13]. We recalculated the data of each study and expressed the marker performance as sensitivity and specificity. BSP, DSPP, OPN, MMP2, MMP3 and MMP9 revealed more than 50% of sensitivity, DSPP (89%) illustrating the highest sensitivity [10]. p53 (80%) in one study exhibited high sensitivity and low in another [8,11] eIF4E and OPN exhibited less sensitivity [11,13]. p53, DSPP, OPN, MMP3, MMP9 and eIF4E demonstrated more than 50% of specificity with OPN and MMP9 showing 100% specificity [8,10,13]. BSP, MMP2, MMP3 and p53 in another study lacked specificity [10,11]. Among all the markers studied MMP9 had the highest accuracy at 80% followed by p53 (75%), DSPP (70%) and OPN (70%) [8,10] while eIF4E [11] had least accuracy. Almost all the studies used Fisher's exact and Fisher-Freeman-Halton significance test.

Quality of the included studies

Only one study [10] was at low risk of bias, three studies [8,11,14] were at moderate risk of bias and three studies [9,12,13] had serious risk of bias. The studies with serious risk mostly had missing data which questions the quality of these studies. The remaining one study [1] did not have enough information to calculate the risk of bias. (Supplementary table)

Significance of Potential Markers

Mutation in p53 gene is a well-known genetic abnormality in variety of cancers including HNSCCs [15]. Studies have revealed that p53 gene mutation leads to the pathogenesis of HNSCC cases with 50 to 60 percent of tumor cells expressing the p53 protein [16,17].

Eukaryotic translation initiation factor 4E (eIF4E) has role in initiation of protein synthesis, overexpression of which induces both the transformation and tumorigenesis as well as initiates metastasis [18]. Studies have reported 100% expression of this marker in HNSCC [9,19] which makes it a potential marker to predict recurrence.

Matrix Metalloproteinases (MMPs) have a key role in breakdown of basement membrane (BM), extracellular matrix (ECM) and contribute in release of growth-promoting signals, modulation of immune responses, apoptosis, and neo-angiogenesis, all of which is essential for tumor progression and growth [20]. The MMP-2 and MMP-9 degrades type IV collagen and its upregulation is associated with the degradation of the BM and the ECM, and with an increase in tumor aggressiveness [21]. Studies have revealed that MMP-9 is a prognostic indicator for malignant potential of OSCC [22,23].

Dentin sialophosphoprotein (DSPP), Osteopontin (OPN) and Bone sialoprotein (BSP) are reported to be upregulated in variety of cancers including OSCCs [24]. DSPP has been associated with aggressiveness of OSCCs and it has been expressed in oral premalignant lesions with dysplasia indicating subsequent invasive OSCC [25]. OPN has been coupled with proliferation, cancer cell growth, invasion and metastasis [26].

Beta-2-adrenergic receptor play role in regulation of tumor cell mechanisms including apoptosis, angiogenesis, proliferation, migration and metastasis through catecholamine induced activation under chronic psychological stress [27]. Studies have reported that β 2-AR has role in metastasis of OSCC and it is involved in proliferation and invasion of tumor cells [27,28].

E-cadherin is an inter-cellular adhesion molecule and regulates epithelial cell to cell adhesion [29]. The reduced immuno-expression of E-cadherin is correlated with malignancy, tumor invasiveness and carcinogenesis [30].

DISCUSSION

Currently, the value of the use of immunohistochemical markers in routine examination of surgical margins of HNSCC has not yet established. More should be known about the analytical validity of the marker used, regarding accuracy and reliability.

This systematic review was an attempt to synthesize the existing data on prognostic role of immunohistochemical markers in surgical margins of patients with HNSCC. The total of eight studies met our inclusion criteria. Since included studies had heterogeneity in intervention (duration, nature, and content), inclusion and exclusion criteria, setting, duration of follow-up and methods of outcome assessment, we could not undertake meta-analysis. However, we have unambiguously offered the details and findings of the included studies.

Very few studies and diversity of evidence restrict the deductions that we can draw in this review and are deficient for definitive evidence. Limited data from very few included studies in this systematic review confirms the need for further large randomized control trials with long-term follow-up focusing on the evaluation of more markers and combination of markers. We look forward to such trials especially for oral squamous cell carcinoma patients.

There are varied IHC markers which could be used for detection of residual cancer in surgical margins of HNSCC patients. The studies included here used eIF4E in HNSCC patients; MMP2, MMP3, MMP9, DSPP, BSP, OPN, Beta-2-adrenergic receptors and E-cadherin in OSCC patients while p53 in both HNSCC and OSCC patients. The results were inconsistent in different studies due to methodological flaws, different sample size and follow-up time.

One study analyzed eIF4E in histologically negative surgical margins by western blot method; they used eIF4E antibody by immunohistochemistry in one case who had recurrence and elevated levels of eIF4E in western blot analysis[9]. Another study that analyzed eIF4E and p53 kept a 5% cut off value on the basis of expression of marker in basal cell layer of surgical margin and compared the expressions of the antibody concluding that the eIF4E is a better predictor of residual cancer compared to p53[11]. A prospective, observational and bilateral study in Australia and India was conducted using eIF4E and p53 concluding eIF4E to be a better prognosticator[1]. While, a study predicted p53 a useful biomarker as there is 5.333 times greater chance of recurrence in a p53 positive surgical margin[8].

DSPP, BSP and OPN are three members of the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) family of proteins and a study [10] reported that 45% of histologically negative surgical margins of OSCCs express at least one of the SIBLINGs and one of the MMPs (MMP2, MMP3, MMP9). However, only DSPP, OPN and MMP9 exhibited

significant association with recurrence or recurrence free survival. When sensitivity, specificity and predictive value were considered, MMP9 had the greatest overall accuracy. While another study that evaluated E-cadherin and MMP9 expression at negative surgical margins of OSCC, suggested that E-cadherin is the preferred marker over MMP9 [12]. A study which evaluated only OPN documented that the 55% of tumor free surgical margins showed positive expression of OPN in the epithelium, inflammatory cells and stromal cells which can be correlated to local recurrence and a poor prognosis[31].

A single study was found that evaluated β 2-AR expression in the tumor free surgical margins of OSCC patients, but no statistically significant correlation was found with clinical variables and elevated levels of β 2-AR [14].

The studies mainly conducted qualitative analysis of the IHC staining. Only one study did quantitative analysis using Intensity Reactivity Score (IRS) which is calculated by multiplying Staining Intensity (SI) with percentage of positive cells (PP) [12]. One study did semi-quantitative analysis depending upon the percentage of positive cells [10]. A study used MATLAB computing language-based software and the representative images were segmented using the software and analysis was done. The median was established as cut off value to classify the intensity of staining [14].

The studies used log rank test and a Kaplan-Meier curve to analyze the probability of local recurrence of cancer [9,10,11]. Univariate analysis and Multivariate Cox regression analysis was used when multiple markers were analyzed for their significance in recurrence free survival of the patients [10,11]. Contingency tables and χ^2 -test were used to evaluate the association of molecular markers with clinical characteristics [10]. Wilcoxon, Mann-Whitney, Kruskal-Wallis, Spearman's rank correlation coefficient test and Fisher's exact tests were used to assess the difference between expression of markers and clinico-pathological parameters in the studied groups [12,14].

There are certain limitations that should be taken into consideration in this systematic review. The studies had different inclusion and exclusion criteria, a study [10] included margins of patients with metastatic lymph node while in another study [11] such patients were excluded. A study included surgical margins with presence of dysplasia as well which should have been excluded [8]. The main limitation being the confounding factors and selection of participants as reflected by ROBINS-I tool of assessment. The results varied from low to critical bias in confounding factors and low to serious risk of bias in selection of participants (Supplementary table). The wide heterogeneity of these studies regarding varied group of immunohistochemical markers in different population for diverse follow up time is an important limitation. Such variable factor does not allow creating

a standard prognostic marker for cancer patients. Therefore, further studies with enough and elaborative data regarding immunohistochemical markers with large follow up time and large number of samples is recommended.

CONCLUSION

In summary, the study shows that immunostaining of resected margins for tumor markers can significantly contribute to the field of head and neck carcinomas. Future efforts should concentrate on improving the antibody selection and its performance in the patients with detailed information. Long term studies with different panel of tumor markers, their qualitative analysis with survival data can help in establishing a potential prognostic marker which can be used in surgical margins of HNSCC and OSCC.

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Table 1: Summary of the characteristics of included studies

Study	Author	Year	Country	Recruitment period	Number of patients	Age range (yr)	Tumor site	Specimen analyzed	Detection method	Follow-up time (months)
1	Ball ⁸	1997	USA (Indiana)	1988-1992	24	65 (mean)	OSCC & OP	Tissue	IHC	24
2	C-A O ⁹ Nathan	1997	USA (Louisiana)	ND	23	45-75	HNSCC	Serum Tissue	Western blot and IHC	7.8-13.6
3	Ogbureke ¹⁰	2012	USA (Georgia)	January 2004-December 2007	20	33-85	OSCC	Tissue	IHC	1-36
4	Jagtar Singh ¹¹	2013	Australia	2006-2009	24	46-81	HNSCC	Tissue	IHC	1-74
5	Nooshin Mohtasham ¹²	2014	Iran	2000-2012	58	32-77	OSCC	Tissue	IHC	ND
6	Vijaya Nirmala Subramani ¹³	2015	India	ND	20	ND	OSCC	Tissue	IHC	ND
7	Oliveira ¹⁴	2016	Brazil	1970-2000	50	≤58 and >58	OSCC	Tissue	IHC	ND
8	Joseph S ¹	2017	Australia and India	November 2013-November 2015 and September 2014-September 2016	50	ND	HNSCC	Tissue	IHC	12

*HNSCC- Head and Neck Squamous Cell Carcinoma; IHC-Immunohistochemistry; ND- Not described; OP- Oropharynx; OSCC- Oral Squamous Cell Carcinoma

Table 2: Parameters of immunohistochemical markers used in included studies

Study	Antibody with source	Source	Dilution	Control	Expression
1	p53 ⁸	Oncogene Science	ND	ND	Colorized product in nucleus and/or cytoplasm
2	eIF4E ⁹	ND	1:500	Zenker's Diverticulum	Blue/Purple
3	MMP2, MMP3, MMP9 DSPP OPN BSP ¹⁰	Santa Cruz Biotechnology	1:100	ND	Reddish brown
4	p53 eIF4E ¹¹	Dako Abcam	1:200 1:500	ND	Brown nuclei Reddish brown peri-nuclear cytoplasm
5	MMP-9 E-cadherin ¹²	Novocastra	1:20 1:50	ND	Brown Cytoplasm Brown Cell membrane and nuclei
6	Osteopontin ¹³	Abcam	1:250	Human colon carcinoma	Dark brown membranous and cytoplasm
7	Beta-2 adrenergic receptor ¹⁴	Santa Cruz Biotechnology	1:50	Vascular smooth muscle	Brown plasma membrane and cytoplasm
8	p53 eIF4E ¹	Ventana	Pre-diluted 1:500	Standardized in lab Breast carcinoma	Brown nuclei Brown peri-nuclear staining

*BSP- Bone Sialoprotein; DSPP- Dentin Sialophosphoprotein; eIF4E-Eukaryotic Translation Initiation Factor 4E; MMP-Matrix Metalloproteinase; OPN- Osteopontin.

Table 3: Immunohistochemical markers with potential to predict the recurrence in HNSCC and OSCC

Marker	Patients and Tumors	Samples (total number studied)	Results (No. of positive/total)	No. of Recurrence
p53 ⁸	OSCC + OP (N=24)	Tumor free surgical margins (N=24)	14	10
p53 ¹¹	HNSCC (N=24)	Tumor free surgical margins (N=24)	13	3
eIF4E ¹¹	HNSCC (N=24)	Tumor free surgical margins (N=24)	21	6
MMP2 ¹⁰	OSCC (N=20)	Negative resection margins (N=20)	12	9
MMP3 ¹⁰	OSCC (N=20)	Negative resection margins (N=20)	13	9
MMP9 ¹⁰	OSCC (N=20)	Negative resection margins (N=20)	6	9
MMP9 ¹²	OSCC (N=58)	Tumor free surgical margins (N=58)	ND	ND
DSPP ¹⁰	OSCC (N=20)	Negative resection margins (N=20)	13	9
OPN ¹⁰	OSCC (N=20)	Negative resection margins (N=20)	11	9
OPN ¹³	OSCC (N=20)	Tumor free surgical margins (N=20)	11	1
BSP ¹⁰	OSCC (N=20)	Negative resection margins (N=20)	13	9
E-cadherin ¹²	OSCC (N=58)	Tumor free surgical margins (N=58)	ND	ND
Beta-2-adrenergic receptor ¹⁴	OSCC (N=62)	Tumor free surgical margins (N=62)	50	26

*BSP- Bone Sialoprotein; DSPP- Dentin Sialophosphoprotein; eIF4E-Eukaryotic Translation Initiation Factor 4E; HNSCC-Head and Neck Squamous Cell Carcinoma; MMP-Matrix Metalloproteinase; OPN- Osteopontin; OP- Oropharynx; OSCC- Oral Squamous Cell Carcinoma

Table 4: Performance of immunohistochemical marker in patients with and without recurrence during follow-up.

Marker	Patients and tumor free surgical margin	Prevalence (no. of patients with positive IHC margin/total no. of patients)	Sensitivity (no. of positively tested/ no. of recurrence case (%))	Specificity (no. of negatively tested / no. of cases without recurrence (%))	Accuracy (%)	Significance* (test)
p53 ⁸	24	14/24	8/10 (80)	10/14 (71)	18/24 (75)	Fisher's exact test and sample odds ratio test
p53 ¹¹	24	13/24	3/13 (23.1)	4/11 (36.4)	7/24 (29.2)	Fisher's Exact tests
BSP ¹⁰	20	9/20	6/9 (67)	4/11 (36)	10/20 (50)	Fisher's Exact and Fisher-Freeman-Halton test
DSPP ¹⁰	20	9/20	8/9 (89)	6/11 (55)	14/20 (70)	Fisher's Exact and Fisher-Freeman-Halton test
OPN ¹⁰	20	9/20	7/9 (78)	7/11 (64)	14/20 (70)	Fisher's Exact and Fisher-Freeman-Halton test
OPN ¹³	20	11/20	1/11 (9)	9/9 (100)	10/20 (50)	Pearson chi-Square test
MMP2 ¹⁰	20	9/20	5/9 (56)	4/11 (36)	9/20 (45)	Fisher's Exact and Fisher-Freeman-Halton test
MMP3 ¹⁰	20	9/20	7/9 (78)	5/11 (45)	12/20 (60)	Fisher's Exact and Fisher-Freeman-Halton test

Table 4: (Continued)

Marker	Patients and tumor free surgical margin	Prevalence (no. of patients with positive IHC margin/total no. of patients)	Sensitivity (no. of positively tested/ no. of recurrence case (%))	Specificity (no. of negatively tested / no. of cases without recurrence (%))	Accuracy (%)	Significance* (test)
MMP9 ^o	20	9/20	6/9 (67)	10/10 (100)	16/20 (80)	Fisher's Exact and Fisher-Freeman-Halton test
eIF4E ¹¹	24	21/24	6/21 (28.5)	2/3 (66.6)	8/24 (33.3)	Fisher's Exact tests

*BSP- Bone Sialoprotein; DSPP- Dentin Sialophosphoprotein; eIF4E-Eukaryotic Initiation transcriptionFactor 4E; IHC-Immunohistochemistry; MMP-Matrix Metalloproteinase; OPN- Osteopontin.

Supplementary Table: ROBINS-I assessment tool-based quality analysis of the included studies.

Study	Confounding bias	Selection of participants bias	Classification of interventions bias	Deviations from intended intervention bias	Missing data bias	Measurement of outcomes bias	Selection of the reported results bias	Overall Risk of Bias
Ball ⁸	Low	Moderate	Moderate	Low	Moderate	Low	Moderate	Moderate
C-A ONathan ⁹	Critical	Moderate	Moderate	Low	Moderate	Moderate	Moderate	Serious
Ogbureke ¹⁰	Low	Low	Moderate	Low	Low	Moderate	Moderate	Low
Jagtar Singh ¹¹	Moderate	Low	Moderate	Low	Low	Moderate	Moderate	Moderate
Nooshin Mohtasham ¹²	Moderate	Moderate	Moderate	Low	High	Moderate	High	Serious
Vijaya Nirmala Subramani ¹³	Moderate	Serious	Moderate	Low	High	Moderate	Moderate	Serious
Oliveira ¹⁴	Low	Low	Moderate	Low	Moderate	Moderate	Moderate	Moderate
Joseph S ¹	Serious	Moderate	Low	NI	NI	NI	NI	NI

*NI- No Information

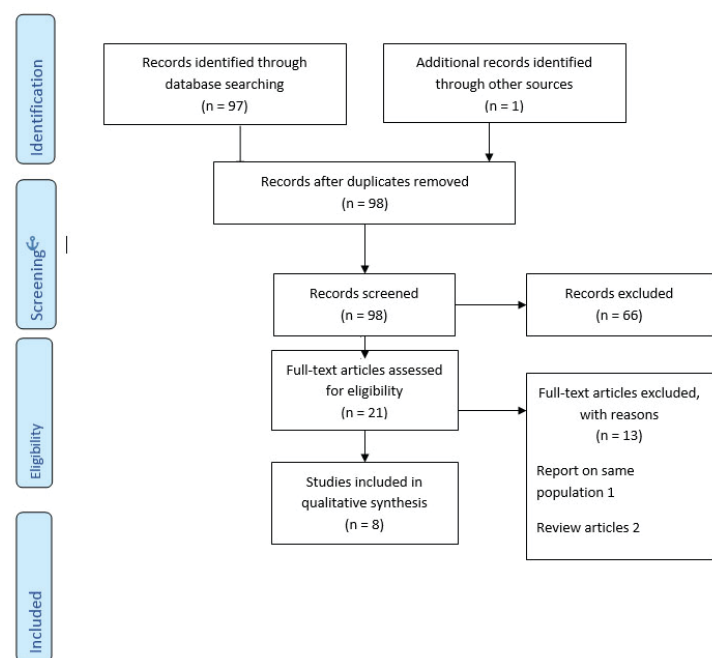


Figure 1: Flow chart of methodology according to PRISMA guidelines