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Role of Immunohistochemical Markers in Evaluating Malignant Transformation of Oral Submucous Fibrosis: A Systematic Review

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***Abstract*---**Objective(s): We present a systematically to identify, evaluate and assess the role of Immunohistochemical markers in Malignant Transformation of Oral Submucous Fibrosis (OSF). Materials and Methods: Extensive literature search was done to identify eligible through "MEDLINE"/ "PubMed", "Scopus" and "Cochrane library" were searched for relative studies until December 2021. The included studies were published in English language were mainly retrospective original research\.These studies mainly evaluated the role of Immunohistochemical markers in Malignant Transformation of Oral Submucous Fibrosis. Results: Thirteen studies were included in the present study which had total of 549 cases. Most of the studies suggested use of combined biomarker model or panel of antibodies to minimize the risk of bias. Almost all the studies used ANOVA and chi-square test while kappa test analysis for interobserver

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variability test. Conclusions: The significance of immunohistochemical markers has been shown in study which can significantly contribute in diagnosing early event in process of malignant transformation of suspicious OSF cases so that better treatment plan could be formulated for better prognosis. More efforts should be emphasized on combination of antibodies and its importance in target drug therapy.

***Keywords*---**COX2, e-cadherin, oral submucous fibrosis, α-SMA, β-catenin.

Introduction

In the last few decades “Oral submucous fibrosis (OSF)” has emerged as a challenge in terms of therapeutic interventions especially in high risk cases. It is known to have to have a high probability of transforming into malignancy that is OSCC as it is a potentially malignant oral disorder. It has a high prevalence rate in India along with many western countries. Oral mucosa is mostly effected in early and moderate stages. In advance stages the parts involved are “pharynx” and “upper third of the esophagus .Mostly the blanching of mucosa is manifested as the earliest clinical feature which is succeeded by intolerance to hot and fiery food and later rigidity of various mucosa ultimately leading to very limited opening of mouth and very less movement of tongue. The histopathology of biopsied specimen shows thinning or atrophy of epithelium, hyalinization of juxtraepithelial area ultimately leading to fibrosis. The pathophysiology of this disease has been explained through various theories (Sudarshan & Annigeri, 2012). Various signaling pathways have been suggested for the fibrosis. The cause of this fibrosis is mainly due to chewing of arecanut which releases arecoline (alkaloid) causing abnormal increase in production of collagen and tannins and catechins (flavonoid) which has effect on breakdown of collagen.

The malignant potential of “oral submucous fibrosis” has been estimated to be 7-13% by “Paymaster” in 1956. They are precursors for OSCC. Thus early detection of cancer initiation and disease progression will be crucial in decreasing incidence of OSCC which will effect the survival of patients. Early detection of disease progression can best be studied at molecular level even before the clinical changes are evident. For understanding the multiplex process of malignant transformation of this disease, copious efforts have been made by using ancillary screening aids like molecular biomarkers, salivary diagnostics and in researches which can give more improved accuracy (Ekanayaka & Tilakaratne, 2016). Main focus of studies conducted in past few decades on epithelial dysplasia and fibrosis in OSF. But very few studies have been conducted to study the early events in malignant transformation of OSF even before its clinical appearance. Definitive and satisfactory treatment intervention methods plays a crucial role improving the prognosis, which are still questionable. With the help of the research studies, we could be able to detect early event in process of malignant transformation of OSF at molecular level ,This will contribute in formulating a target drug therapy which will definitely benefit the patient.

Summing it up, early detection of potentially malignant OSF and understanding the mechanism involved in the process will play a role in inhibition of OSCC. Ultimately targeting these molecules to design and plan the standard treatment modalities for high risk OSF cases with hope for better prognosis. To assess the role of immunohistochemical markers in the process of malignant transformation of Oral Submucous Fibrosis (OSF), till date, no study has been systematically evaluated. We have followed the preferred Reporting for Systematic Reviews and Metanalysis in this research (Moher et al., 2010).

Method

Eligibility Criteria

The following criteria was used for chosen studies.

Study design

- Included: Original research studies which were mainly retrospective, from the date of inception to December 2021."
- Excluded: All the "case series", " animal studies", "review papers", "conference papers, "abstracts" and "unpublished data",

Intervention

- Included: immunohistochemistry (IHC) studies.
- Excluded: "RT-PCR", "DNA methylation", "gene analysis", used in studies

Participants

- Included: Patients diagnosed with "OSF" and "OSF" with coexisting "OSCC "\.
- Excluded: Patients with OSCC without pre-existing OSF and individuals suffering from collagen disorders were excluded.

Outcome

The value of immunohistochemical markers to understand the molecules molecular involved in the early event in the process of malignant transformation in OSF and improved prognosis of high risk OSF cases.

Search strategy

The Mesh terms used for search are ("OSF"), or ("OSF with OSCC") and ("Markers For Malignant Transformation")and extensive literature search was carried in "MEDLINE"/"PubMed", "Scopus" and "Cochrane library" to identify pertinent articles. All the retrived studies were thoroughly scanned along with their citations to recognise other possible relevant studies. These were again evaluated to exclude any unrelated publications.

Data extraction

Two authors created a systematic data extraction sheet to exclude irrelevant articles. Based on the inclusion and exclusion criteria, the included articles were evaluated and included in this sheet .A third author was there to resolve the disagreement arising in these two steps. This data sheet was used to extract data from relevant studies under categories: “author”, “year”, “country”, “recruitment period”, “number of patients”, “tumor type”, “specimen analyzed”, “detection method”, “antibody source”.

Results

Search results and outcome

A total of 23 articles were found, in the initial search strategy. After screening of the titles and abstracts, eleven articles who included methods other than immunohistochemistry were exclude. Out of twelve articles four articles were review, four were serum-based studies, two articles included studies with routine Haematoxylin and eosin method and one included nucleotide array. Finally twelve full text articles met the criteria of the present review and were included in it.

General characteristics of eligible studies

The total number of cases were 549, all articles were from Asian ethnicity. In all articles there were cases of OSF, OSCC Nithya et al. (2019); Divyambika et al. (2021); Joseph et al. (2020); Bazarsad et al. (2017); Patel et al. (2015); Bavle et al. (2020); Hosur et al. (2021), and in three articles has cases of OSCC in background of OSF Monteiro et al. (2021); Quan et al. (2020); Raju K et al. (2020), and two articles used cases of oral potentially malignant disorders (Sharada et al., 2018; Chowdhury et al., 2021). All were retrospective studies except one which was prospective study. All were analysed histopathologically and then immunohistochemistry was done. One study included immunohistochemistry which was correlated with immunofluorescent staining and PCR.

Immunohistochemical markers related parameters of included studies

There are diverse variety of “IHC markers” that can be used either alone or in cocktail to detect malignant potential of OSF. The studies included here used Loricrin, Cyclooxygenase 2, Hypoxia-inducible factor- 2α, “Ki67”, “ cyclin D1”, “p16,” “p53”, “b-catenin”, “c-Jun”,” c-Met”, and “insulin like growth factor II mRNA-binding protein 3” “(IMP3)”, E-cadherin and VEGF , COX-2, p53, and MDM2 , p63 and CD31 , E-cadherin, Twist1 and Snail1 in OSF patients ,while β-catenin in oral leucoplakia, OSF, PTEN and “α- SMA” in “OSF” and “OSCC ” with concomitant “OSF”, Programmed death ligand-1 in“ OSCC” with or without OSF. hTERT in OSF and OSCC. (Table 2) Loricrin was used in one study at a dilution of 1:500 from MERCK with foreskin as positive control. Cellular staining was localized in both cytoplasm and nucleoplasm as brown staining. Cyclooxygenase 2 (COX-2) was included in two studies, one study used COX-2 from BioGenex. Positive cells were stained as brown in epithelium and connective tissue.

In other study combination of “COX-2”, “p53”, and “MDM2” was used, In that COX-2 and MDM2 at a dilution of 1:100 from “Leica Novocastra” (“Newcastle upon Tyne, UK”) was used and p53 was ready to use. “Ulcerative colitis” for “COX-2”, “colon carcinoma” for “p53 “, and “osteosarcoma” for“ MDM2” were used as positive controls. COX-2 exhibited cytoplasmic staining, while p53 and MDM2 stained the nucleus. Hypoxia-inducible factor -2α was used in one study was used at a dilution of 1:100 from Abcam. Preeclamptic placental tissue was used as positive control. In normal oxygen conditions, “HIF-2α” expression is seen in cytoplasm only, whereas in low oxygen conditions, both the cytoplasm and in the nucleus show the expression as brown staining. “Ki67”,” cyclin D1”, “p16,” “p53”, “b-catenin”, “c-Jun”,” c-Met”, and “insulin like growth factor II mRNA-binding protein 3” “(IMP3)”were used in one study in which the dilution used for “Ki67”,” p53”, and “c-Jun” were used in 1:100, 1:400 for “cyclin D1”, 1:50 for “IMP3”, and 1:80 for “b-catenin”, respectively, from “Abcam” “(Cambridge, UK”); “p16” was used in 1:50 and “c-Met” was used in 1:200, from “Santa Cruz Biotechnology”.

Ki67 and cyclin D1 were stained as brown in nucleus, p16 stained brown in both nuclear and cytoplasmic, IMP3 showed epithelial cells with cytoplasm and perinuclear immunoreactivity. β-catenin showed membranous immunoreactivity. p53 showed nuclear expression in basal cell layer of epithelium. c-Met stained cytoplasm. of basal and suprabasal layer of epithelium and “c-Jun” showed immunoreactivity in nucleus of basal cell layer.[7] E-cadherin was used in two studies. In one study E-cadherin was used with VEGF according to the manufacturer’s protocol (Pathnsitu). Breast carcinoma was used as positive control for E-cadherin and OSCC was used as positive control for VEGF. It gave brown membranous staining for E-cadherin and brown cytoplasmic staining for VEGF. Other study used E-cadherin, Twist1 and Snail1 from Dako and stained in private lab. Normal oral mucosa was used as control group. E-cadherin showed immunoreactivity in cell membrane, Snail 1 showed immunoreactivity in nucleus and sometimes cytoplasm whereas Twist staining was evaluated by extent and intensity. p63 and CD31 from Biogenex were used in one study. Both showed immunoreactivity to epithelium. β- catenin PathnSitu Biotechnologies was used in one study with breast carcinomas as a positive control.it showed immunoreactivity as a brown stain in membranous, cytoplasmic, nuclear region as well as in layers of epithelium. PTEN and α- SMA from BioGenex Laboratories was used in another study and it stained nucleus and cytoplasm Brown for both PTEN and α-SMA expression respectively. Programmed death ligand-1 from Abcam was used at a dilution of 1:50 in a study. where % of positive cells between 0 to 25 % was considered low whereas ≥25%of tumor infiltrating lymphocytes were considered as high hTERT was used in one study at a dilution of 1:25 from Biogenex. It gave brown stain to nucleus and cytoplasm.

Immunohistochemical studies: Potential markers

Formalin fixed paraffin embedded and archived blocks of “OSF” or “OSF” with concomitant “OSCC” were included in the studies. hTERT was used in 50 cases (20 OSF, 20 OSCC, 5 OSCC with concomitant OSF) out of which showed significant increase in 25 cases(OSCC & OSCC with concomitant OSF). Programmed death ligand-1 was used in 54 cases (44 OSCC with concomitant OSF, 10 OSCC) out of which OSCC with concomitant OSF showed significant

increase. E-cadherin was used in two separate studies, in one study 60 cases (progressive grades of Oral Epithelial Dysplasia, OSF, OSCC were used), out of which severe oral epithelial dysplasia and OSCC. In this study VEGF was also used and also showed similarresults.

In other study E-cadherin was along with Twist and Snail in 40 cases (OSF with and without dysplasia and normal mucosa) in which no significance was shown in all markers between different groups. β-catenin was used in two studies. In one study it was used in 40 cases (different grades of dysplasia, OSF). It showed increased expression from mild to severe dysplasia to OSF. In other study , β-catenin was used in combination with other markers[["Ki67," "cyclin D1", "p16", "p53," " c-Jun", "c-Met", and" insulin like growth factor II mRNA-binding protein 3" ("IMP3")in 36 cases ("OSF"). The expression of "Ki67", "cyclin D1", "c-Met", "IMP3", and "b-catenin" was statistically significant difference between study groups whereas the combined expression of "Ki67" and "p16" were statistically different expression between the "transformation" and "non-transformation" groups. The expression of COX-2 was seen in two studies. In one study, COX-2 was used in 70 cases (10 "normal mucosa", 30 "OSF" and "OSCC" with "OSF"). The expression of COX-2 was elevated in different stages of OSF a compared to normal oral mucosa. COX-2 was used in another study in combination with p53 and MDM2 in 40 cases (early OSF, advanced OSF, OSCC with coexisting OSCC, OSCC), expression of all three elevated with disease progression.

PTEN and α-SMA was used in 70 cases (10 normal mucosa, 30 OSF, 30 OSCC with coexisting OSF). PTEN immunoreactivity was reduced and α-SMA expression was increased from normal mucosa to OSF and OSCC. In one study, Hypoxia-inducible factor-2α was used in 41 cases 11 OSF, 15 OSCC with arecanut chewing, 15 cases of OSCC without arecanut chewing. Significant expression was seen in all cases. Loricrin expression was seen in one study in 72 cases (11 normal oral mucosa, 32 hyperkeratotic, 30 OSF). Its expression was observed in all cases of OSF associated with habits. The combination of p63 and CD31 was used in 36 cases (early OSF, moderate OSF, advance OSF). A significant increase in staining of p63 and CD31 expression was observed through different stages of OSF.

Significance of potential markers

hTERT (human Telomerase reverse transcriptase) Telomerase basically prevent the shortening of telomeres. It's catalytic subunit hTRET shows increased activity in oral potentially malignant disorders and OSCC.so it can play a role as diagnostic marker to predict malignant transformation in high risk cases of OSF. Loricrin are the proteins of Cornified epithelial Envelope expressed in large quantities in keratinocytes which are differentiated due to stress (mechanical or chemical).so its expression in large quantities in OSF cases could indicate an early event in process of malignant transformation in high risk cases.

Hypoxia-inducible factor-2α ("HIFs- 2α") regulates the low oxygen conditions {hypoxia) arising due to Hypoxia is induced by arecanut chewing and fibrosis. Tumor growth. Progression, genes, genes, "angiogenesis," "glycolytic metabolism" etc are regulated by them. Their involvement in the process of malignant

transformation have been shown in cases of “breast” and “prostrate carcinomas” and in “OSF”.PTEN (phosphatase and tension homolog deleted on chromosome 10) idownregulates cell growth and survival. It negatively regulates “phosphatidyl inositol-3-kinase”/”AKT” (“PI3K/ AKT”) pathway thereby controlling myofibroblast differentiation in fibrotic disorders. Similar evidences has been reported in thyroid, kidney, lung, breast; endometrial precancer malignancies. It acts as dual phosphatase by acting as tumor suppressor as well as metabolic regulator, the absence of “phosphatase” and “tensin” homologue positivity in “OSF” cases suggests that these cases are high risk cases and may have greater probability of turning into malignancy and may act as prognostic marker on disease progression.

β-catenin belongs to “armadillo” family of proteins .Its main localization in in subcellular areas, playing a crucial role in homeostasis and developmental process, stabilizes cell-cell contacts and also in “cytoplasm”. Being a fundamental of “Wnt “pathway, it regulates cellular activities like “proliferation”, “polarity” and determines “fate” during “embryonic development”. Mutation in the “Wnt” pathway is often correlated to “human birth defects”, “cancer” and other diseases. With the increase in severity of the dysplasia, there is an “translocation” of β-catenin from its primary site to final destination i.e., nucleus leading to trigger in its proliferation. This translocation points to increase in “dedifferentiation”, ultimately pointing to the cancerous potential in the highly suspicious cases of “OSF”.

c-jun is a basic leucine zipper transcription factor that regulates gene transcription. It is involved in various cell activities like cellular proliferation, cell death, its survival as well as carcinogenesis and lastly morphogenesis. p63 (homolog of p53) is a transcriptor regulator with a crucial role in cell proliferation, differentiation and maturation. It is frequently altered in epithelial dysplasia and increased in OSCC and often associated with carcinogenesis. CD31 is an endothelial marker of immunoglobin super family its expression is varied in epithelial dysplasia and OSCC. So epithelial alterations along with underlying angiogenic support is vital process in detfor ermining the conversion of OSF into OSCC.

Programmed death-1ligand acts as immune checkpoint blockade. It plays a vital role in tumor escape from immune response and has been found to be very effective in melanomas and non-small cell lung cancer .Its expression is upregulated in OSCC in background of OSF. E-cadherin or cadherin 1 is a transmembrane glycoproteinan that act as “inter-cellular adhesion molecule”. It is mainly responsible for various cellular functions like “cell to cell adhesion”, “polarity”, “differentiation”, “migration” Downregulation of E-cadherin is associated with loss of differentiation, tumorigenesis, and invasiveness of cancer cells. Being the member of “platelet-derived growth factor” (“PDGF”) family VEGF (Vascular Endothelial Growth Factor), stimulates endothelial cells proliferation leading to new vessels formation. This is one of the major event in “tumorigenesis” and “metastasis”.

Twist isa transcription factor. It serve as a transcription repressor to activate EMT traits by repressing the expression of E-cadherin. Upregulation of Twist with

downregulation of “E-cadherin” expression plays a vital role in tumor progression. It has been recognized in various epithelial tumors. It is also seen in pulmonary, liver and kidney fibrosis. The chemicals released by arecanut chewing causes upregulation of fibroblasts in buccal mucosa whereas there is downregulation of TWIST by lentiviral knockdown resulting in reversal of myofibroblastic differentiation thus suggesting its role in pathogenesis of OSF. Snail is a master gene in regulating various aspects of the EMT phenotype .This includes excess expression of mesenchymal markers like fibronectin, vitronectin and also suppresses E-cadherin. On the relation between Snail expression and EMT can be focussed in several types of cancers like OSCC.” The physiological function of “α-SMA “tissue growth”, “development” and “repair”. They secrete excessive extracellular matrix protein in response to tissue injury. If expressed in tissue, it is due to the presence of myofibroblasts but it is also expressed in various grades of intensity in different grades of “OSF” and “OSF” with coexisting “OSCC”. Its expression is correlated with tumor formation and progression.

“IMP3” has been evaluated in recent studies showing its involvement in “tumor cell proliferation” and “invasion”. As it is an “oncofoetal “protein it may play a vital role in tumor initiation and progression. During embryonic development, a vital role in morphogenic organization is played by c-Met ‘it controls the structure and function of adult tissues, including “cell migration” and “proliferation”. It is also essential for repair of injury. “c-Met” expression have been shown to be significantly increased in cases of the “transformation” from “NOM” to “epithelial dysplasia” and to “OSCC”. The gene on chromosome 10q25 mainly encodes Ki-67 .All the phases of cell cycle marks the expression of Ki-67, G0 phase and early G1 phase marks the exception. The expression increases in S phase with a peak in G2 and M phase, and then down regulates after mitosis. Researchers conducted have shown increased expression of Ki_67 in the suprabasal epithelium. This increases with the severity of dysplasia.

Cyclin-D1 is well known to be susceptible to the influence of various mitogenic stimuli and hence known to be increased in various neoplasm. Many Studies have shown a substantial increase of cyclin-D1 expression with increasing grades of dysplasia and malignancy. Few studies have been conducted in OSF which ll showed varied expression. The tumor suppressor gene p16INK4a is present on chromosome 9P21 and inactivation of p16 gene causes altered expression of p161NK4a (p16) and is considered to be a significant event in the development of many tumors including OSCC.A significant correlation has been reported by several authors between the degree of dysplasia and expression of p16 in dysplastic epithelium, but few reported that p16 is not a useful marker for differentiating between (dysplastic and nondysplastic lesion.

COX-2(Cyclooxygenase 2) is an inducible isoform and immediate early-response gene which is generally more expressed and triggered in the presence of inflammation (as It is regulated by different cytokines such as IL1β, IL6, and TNFα), growth factors, oncogenes, carcinogens, tumor progression. So inflammation initiates an uncontrolled, uncoordinated proliferation of the initiated tumor cells by triggering the production of reactive oxygen species. Many research have shown overexpression of COX 2 in oral cancer which has been linked to tumor vascularization, metastasis, and prognosis. Degradation or

mutation of p53 is a well-known genetic abnormality implicated in OSCC pathogenesis. p53 pathways is activated by various cellular stresses, such as “DNA damage “and “oncogenic signals” resulting in cell cycle arrest and apoptosis. Upregulation of p53 as much as 70% in premalignant conditions like OSF suggests malignant transformation. Also if p53 staining is seen more in parabasal region of epithelium then it is correlated with cancer progression. The “MDM2” gene is a proto-oncogene and negatively regulates p53. It is upregulated in 40% to 80% of all OSCC cases. In cancer, p53 pathways is downregulated by the mutation, deletion or inhibition of TP53 gene.

Discussion

Currently, few studies have focussed on importance of immunohistochemical markers. In assessing malignant transformation of Oral Submucous Fibrosis. The accuracy and reliability of the markers used constitute the analytical validity of the marker used, which is of prime importance for targeting and formulating a standard treatment regime for high risk cases of OSF patients in need for better prognosis. The present systematic review was pursued to incorporate the current data on role of immunohistochemical markers immunohistochemical markers in malignant transformation of oral submucous fibrosis. Our inclusion criteria was fulfilled by the total of twelve studies. Meta-analysis, animal studies, review papers, conference papers, abstracts , “RT-PCR”, “DNA methylation”, “gene analysis” and “unpublished “data were excluded. We have tried to include unequivocal data of the included studies.

The presence of slender data due to very little studies along with heterogeneity of evidence narrows the inference that can be drawn from the present review. So this substantiates the further need of more studies focusing mainly on combination of markers to indicate cancer initiation and progression in high risk OSF cases which could be targeted for intensive treatment modalities, ultimately leading to better prognosis. Various IHC markers been tried which could be used for predicting possible association of the markers with malignant transformation of OSF. These studies included Loricrin, Cyclooxygenase 2, Hypoxia-inducible factor- 2α, “Ki67,” “cyclin D1”, “p16”, “p53,” “c-Jun”, “c-Met”, “β- catenin” and “insulin like growth factor II mRNA-binding protein 3” (“IMP3”), E-cadherin and VEGF, COX-2, p53, and MDM2, p63 and CD31, E-cadherin, Twist1 and Snail1 in OSF patients, while β- catenin in oral leucoplakia, OSF, PTEN and α- SMA in OSF and OSCC with concomitant OSF, Programmed death ligand-1 in OSCC with or without OSF. hTERT in OSF and OSCC. The results varied due to different sample size and other methodological flaws.

In one study Loricrin was analysed immunohistochemically and increased expression was seen in early and intermediate grades of OSF thus showing potential as a early marker. Cyclooxygenase 2 was used in one prospective study, in which 67.9% cases showed increased expression. In this study it was noted that COX 2 expression significantly increased with the increase in severity of fibrosis. In another study COX 2 was used with p53 and MDM2 in which the expression of all increased with disease progression indicating COX 2 to be an important and portent biomarker for assessing disease progression and MDM2 to be important treatment planning.

Hypoxia-inducible factor- 2α was used in one study, in which statistically significant difference was seen between study groups (OSF and OSCC with arecanut chewing). This may indicate process of cancer initiation and its progression. β- catenin was used in two studies. In one study, it was used along with, “Ki67,” “cyclin D1”, “p16”, “p53,” “c-Jun”, “c-Met”, “β- catenin” and “insulin like growth factor II mRNA-binding protein 3” (“IMP3”). In this study, significant difference between study groups was seen with the expression of “Ki67”, “cyclin D1”, “c-Met”, “IMP3”, and “β- catenin”. The combination of “Ki67” and “p16” .showed significant difference between the transformation and nontransformation group. β- catenin was used in another study which showed increased expression in different grades of dysplasia increasing order and in OSF indicating it to be a promising marker for predicting malignant transformation.

E-cadherin was used along with Twist and Snail in one study. Significant expression of all the three markers was seen. There was increased expression of Twist and Snail along with adjuvant loss of E-cadherin suggesting role of EMT in disease pathogenesis and its progression. E-cadherin along with VEGF (vascular endothelial growth factor) was used in another study in which. E-cadherin expression in OSF was intermediate with moderate and severe dysplasia and VEGF expression was similar to mild dysplasia. Both can be used as combination marker to predict the potential risk for malignant transformation.

In one study p63 and CD31 were used, a significant increase in expression was seen in increasing grades of OSF with p63 whereas CD31 expression showed more dialated vessels in increasing grades of OSF. So both could be used as quantitative predictive biomarkers for malignant transformation of OSF. Another study used the combination of PTEN and α- SMA, which showed decrease expression from “normal mucosa” to” OSF” and “OSCC” with PTEN and gradual increase expression from normal mucosa to OSF and OSCC with α- SMA. This inverse relation between PTEN and α- SMA in OSF cases can indicate disease progression. Programmed death ligand-1was used in one study in which increased expression was in OSCC cases with OSF than in OSCC cases without OSF. Double immunofluorescent staining along with Real-time PCR was also done along with immunohistochemistry. It may be an important role in malignant transformation of high risk cases of OSF.

hTRET was used in one study in which increase in expression was seen from “Normal Oral Mucosa” to “OSF”, “OSCC” with” OSF”, ”OSCC” without “OSF” suggesting involvement in early events in malignant transformation of OSF. Qualitative analysis was conducted in six of the included studies. Three studies did semiquantitative analysis. Two studies included used quantitative analysis using” Intensity Reactivity Score” (“IRS”) which is calculated by multiplying “Staining Intensity” (“SI”) with percentage of “positive cells” (“PP”). Three studies did semi-quantitative analysis depending upon the cellular localization, staining intensity and percentage of positive cells. Another study used quantitative analysis for one marker and qualitative assessment for another two markers. The studies with quantitative analysis also used image analysis software to assess the labelling index which was calculated by dividing number of positive cells by total number of cells and multiplying by 100.

The comparison between staining intensity between groups in different studies were done by one way ANOVA, Student's t-test, Chi-square test," Kruskal-Wallis" and" Pearson's chi square test". "Mann–Whitney U test" or "Student's t-test", the independent sample t test was used to compare intensity within groups and between groups. The studies using combined biomarkers used, logistic regression analyses models, Univariate and multivariate analyses to assess the clinicopathological parameters with malignant transformation and discriminant analysis to identify combined biomarker model. Kappa analysis was done to evaluate interobserver variability. The difference between expression of markers and clinico-pathological parameters in the study groups were assessed by "Mann-Whitney"," Pearson Chi square test", "Kruskal-Wallis", "Spearman's rank correlation coefficient test" and "Fisher's exact tests".

Limitations are always part of systematic review which should be considered. These limitations are mainly due to difference in study groups like many of the studies included in this review had OSF as their main group and compared it with normal oral mucosa while few included OSF, OSCC with OSF and OSCC with different grades of dysplasia, also OSF with oral potentially malignant disorders. One of study compared OSCC with OSF and OSCC without OSF along with normal oral mucosa. There was wide range of difference in sample size. Few had very low sample size and the biomarkers used are also not cost effective. The most important limitation was wide range of immunohistochemical markers used in study (varying from single to combination of markers) in different study groups. It becomes very difficult to create a standard biomarker for predicting cancer initiation and progression in OSF due to such divergence and variability in factors. More elaborate studies in future should be conducted on large sample size and with more focus on combination of biomarkers. So that we can evaluate the disease progression in high risk cases of OSF and evolve more fierce therapy options for improving the profnosis.

Table 1
Summarizes the relevant features of included studies

Study	Author	Year	Country	Recruitment period	No, of patients	"Tumor site"	"Specimen "analysed	Method of detection
1	Nithiya s	2019	India	Archival specimens	73	NM, hyperkeratosis, OSF	Tissue	IHC
2	Catakapatri	2021	India	2018-2019	40	NM*, OSF	Tissue	IHC
3	Immanuel J	2019	India	Archival specimens	51	OSF , OSCC with arecanut chewing, OSCC without arecanut chewing,	Tissue	IHC

4	Shadavlonjid	2017	Sri Lanka	Archival specimens	42	OSF, NM	Tissue	IHC
5	Pratik P	2015	India	Archival specimens 2013	40	**EOSF, ***AOSf, OSCC with OSF, OSCC	Tissue	IHC
6	Radhika M	2020	India	Archival specimens 2015	36	Early, Moderate, Advance OSF	Tissue	IHC
7	Mahadevi R	2021	India	Archival specimens	60	OSF with dysplasia, OSF without dysplasia, NM	Tissue	IHC
8	Roshni M	2021	India	Archival specimens Jan 2018 to June 2019	70	OSF, OSCC with OSF	Tissue	IHC
9	Hongzhi Q	2020	Hunan	Archival specimens 2014-2019	94	OSCC with OSF, OSCC, NM	Tissue	IHC, Double immunofluorescent staining, RT-PCR
10	Lizbeth R	2019	India	Archival specimens Nov 2015-March 2018	50	OSF, OSCC with OSF, OSCC	Tissue	IHC
11	P Sharada	2018	India	Archival specimens	60	Mild, Moderate, Severe ED**** OSF. OSCC	Tissue	IHC
12	Pritha C	2021	India	Archival specimens	40	Mild, Moderate, Severe ED, OSF, NM	Tissue	IHC

*NM : Normal Oral Mucosa
** EOSF : Early Oral Submucous Fibrosis
*** AOSF : Advance Oral Submucous Fibrosis
****ED : Epithelial Dys[lasia

Table 2
Parameters of immunohistochemical markers used in included studies

"Study"	"Antibody"	"Source"	"Dilution"	"Control"	"Expression"
1	Loricrin	MERCK	1:500	Foreskin	cytoplasm and nucleoplasm of cells as brown
2	COX 2	BioGenex	ND	ND	Brown stain in Epithelium and Connective tissue
3	COX 2, p53, and MDM2	Leica Novocastra	"COX-2" and "MDM2" at 1:100, and "p53" ready to use.	ulcerative colitis for COX-2 (,colon carcinoma for p53 and osteosarcoma for MDM2	COX-2 as brown cytoplasmic staining, while p53 and MDM2 stained the nucleus.
4	HIF-2α	Abcam	1:100	Preeclamptic placental tissue	Mainly as brown stain in cytoplasm, but in low oxygen conditions, both the cytoplasm and nucleus stains it
5	" Ki67", "cyclin D1"," p16", "p53", "b-catenin", "c-Jun", "c-Met", and ("IMP3")	Dako, Abcam, Santa Cruz	1:100 was used for "Ki67"," p53", and "c-Jun", "cyclin D1" in "1:400", "IMP3" in "1:50", and "b=catenin "in "1:80", respectively , ; "p16" and "c-Met" were used in 1:50 and 1:200,	ND	Ki67 and cyclin D1 as brown stain in nucleus, "p16" showed both "nuclear" and "cytoplasmic" brown staining, IMP3 showed immunoreactivity in "epithelial cells" with cytoplasm and perinucleaer ." β-catenin" showed membranous immunoreactivity. "p53" showed nuclear expression in "basal cell layer of epithelium". "c-Met" showed expression in basal and suprabasal layer of epitheliumin cytoplasm. C-Jun showed "Nuclear "expression cells in" basal cell layer".
6	E-cadherin and VEGF	Pathnsitu	ND	Breast Carcinoma for E-cadherin, OSCC for VEGF	Membranous brown staining for E cadherin and cytoplasmic staining e for VEGF.
7	E-cadherin,	Private Lab	ND	ND	Cell membrane for E-

	Twist1 and Snail1				cadherin, Snail 1 Nucleus and cytoplasm , Twist by extent and intensity
8	p63 and CD31	BioGenex	ND	ND	Brown stain in epithelium
9	β-catenin	PathnSitu	ND	Breast carcinoma	membranous, cytoplasmic, nuclear brown staining
10	PTEN and α-SMA	BioGenex	ND	ND	Brown precipitate in the nucleus and cytoplasm
11	PD-L1	Abcam	1:50	ND	The percentage of positive cells ≥25% of cells was classified as high and between 0% and 25% as low.
`12	hTERT	Biogenex	1:25	ND	Brown stain in nucleus and cytoplasm

COX 2= Cyclooxygenase 2, MDM2= mouse double minute 2, HIF-2α = Hypoxia-inducible factor-2α, IMP3= insulin like growth factor II mRNA-binding protein 3, VEGF = vascular endothelial growth factor. PTEN = phosphatase and tensin homologue, α-SMA= alpha-smooth muscle actin, PD-L1= programmed death ligand-1, hTERT= human Telomerase reverse transcriptase

Conclusion

The synopsis of this review shows that use of immunohistochemical markers to evaluate the process of malignant transformation in OSF can be of significant importance in cases of high risk cases. Detection at an early stage along with quick and expeditious intervention can definitely improve the quality of life in highly suspicious cases of OSF. Focus in future studies should be on the selection of markers preferably a panel or combine biomarker model on large sample size. So that efforts could be concentrated on target drug therapy in high risk cases of OSF rather than radical treatment in cases of OSF already converting into OSCC which will ultimately benefit the patients.

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