Review Article

Expression of transforming growth factor-β in oral submucous fibrosis: A systematic review

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Abstract

Oral submucous fibrosis (OSF) is a potentially malignant disorder characterised by inflammation and progressive fibrosis. Transforming growth factor-β (TGF-β) has been established as a master regulator of fibrosis in various organs; however, lack of systematic review on expression of TGF-β and its isoforms in OSF restrict the understanding of their behaviour in its pathogenesis. Online electronic databases, such as PubMed Medline, Cochrane Library, Embase, and Scopus, were searched from their respective dates of inception till 31st March 2022. Human studies related to TGF-β expression in histopathologically diagnosed OSF cases, with or without malignant transformation, were included and assessed using a Cochrane risk of bias assessment tool: For non randomised studies of interventions (ACROBAT NRSI). The electronic literature search yielded 394 articles. Of those, ten articles met the inclusion criteria and involved total of 579 OSF patients. The risk of bias (RoB) was low to moderate. These studies demonstrated a significant positive expression of TGF-β and its isoforms in OSF compared to that in normal tissue samples. An increased pan TGF-β expression was observed in the early stages of OSF, and an increased expression of TGF-β1 and TGF-β2 were seen in advanced stages of OSF. Stage wise expression of TGF-β3 has not been discussed in the included studies. No significant relationship was observed between epithelial dysplasia and TGF-β expression in OSF. The distinct pattern in the expression of pan TGF-β, TGF-β1 and TGF-β2 in various stages of OSF indicates their different roles in OSF progression. We believe isoform targeted studies exploring stage wise expression of the marker will open new treatment avenues for OSF.

Keywords: Oral submucous fibrosis, OSF, transforming growth factor-β TGF-β, TGF-β1, TGF-β2, TGF-β3

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INTRODUCTION

Oral submucous fibrosis (OSF) is a chronic, insidious, and potentially malignant disorder that affects the oral cavity and is a major global concern with no elaborate population-based data. The pathogenesis of OSF is obscure and is believed to be multifactorial. However, recent studies have established arecoline in areca (betel) nut as the main aetiological agent of OSF.[1,2] The significant histopathological change in OSF is the increased accumulation of type I collagen within the extracellular

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matrix (ECM) in the subepithelial tissues and an imbalance between matrix deposition and degradation. Studies have shown elevated levels of fibrogenic cytokines such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF-β) in OSF tissues.[3]

TGF-β is a multifunctional cytokine that includes three structurally similar mammalian isoforms (TGF-β1, TGF-β2 and TGF-β3). They belong to the TGF superfamily and are central regulators of cell differentiation, proliferation, migration, gene expression and are therefore implicated in both reparative and fibrotic responses. Studies demonstrated upregulation of TGF-β isoforms in multiple organ fibrosis suggesting its potent fibrosis modulatory effect.[4,5] TGF-β1 and TGF-β2 display potent fibrotic activity,[6,7] while TGF-β3 has more of an anti-fibrotic effect.[8]

Various experimental (cell culture/animal model) and clinical based studies suggest upregulation of TGF-β in OSF.[9] The present study was undertaken to collate and discuss all published work on the expression of TGF-β and its isoforms in the progressive stages of OSF in human tissue samples, as to the best of our knowledge no such systematic review has been conducted till date. Differences in the expression of pan TGF-β, TGF-β1, TGF-β2 and TGF-β3 in different stages of OSF were also comprehensively evaluated. We aim to identify any association of TGF-β expression with a specific OSF stage to further help in the identification of the optimal time for pharmacological intervention.

MATERIALS AND METHODS

Registration and protocol

The systematic review was conducted according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement guidelines.[10] The study was registered with International Prospective Register of Systematic Reviews (PROSPERO) under number CRD42022319970.

Focused question

Is there a difference in the expression of TGF-β and its isoforms in OSF and its malignant transformation?

Eligibility criteria

Inclusion criteria included the following: (i) Human studies related to TGF-β expression in histopathologically diagnosed cases of OSF, with or without malignant transformation; and (ii) all full-length studies. Exclusion criteria included the following: (i) Studies on animal tissues, cell cultures, randomised clinical trials; and (ii) those that included clinically undiagnosed cases of oral epithelial dysplasia. Animal experiments are often differently designed, conducted, and analysed; additionally, replication of these results as well as summarisation of evidence from animal research are methodologically inadequate. Human cell culture studies also possess certain limitations: Human cell lines are usually derived from tumours and thus, there is a restricted variety of available cell types; problems of short longevity; and loss of specialisation in culture.[11,12]

Search strategy

To determine if there is a change in expression of TGF-β in OSF and its malignant transformation, a comprehensive literature search was undertaken. Without language restrictions, online electronic databases such as PubMed-Medline, Cochrane Library, Embase and Scopus were searched from their respective dates of inception until March 31, 2022. We investigated supplementary sources like Google Scholar, Livivo database, unpublished papers, conference proceedings and cross-references. Contact was established with authors to procure unpublished studies. To restrict our search results to human research, we incorporated an extra filter. We also searched for relevant publications in oral medicine, oral surgery and oral pathology journals. "Transforming growth factor-beta", "oral submucous fibrosis", "immunohistochemistry (IHC)" and "transformation" were the primary search phrases, which were adjusted according to the glossaries of each database and merged using Boolean operators. One of three researchers conducted the investigation. Figure 1 provides a detailed search strategy for the PubMed database, which was adapted to other databases as needed [Supplementary Table 1].

Screening and selection

We imported all search results into EndNote 20 and reimported all titles and abstracts into the Excel screening workbook. Two researchers independently scanned the papers, first by the title and abstract. Reviews, commentaries, or clinical trials were not included in the search. If the search keywords were present in the title and abstract, the papers were selected for full-text reading. Papers without abstracts, but with titles suggesting that they were related to the objectives of this review, were also selected to screen the full text for eligibility. After selection, the full-text papers were meticulously read by two researchers. Those papers that fulfilled all of the selection criteria were selected for data extraction. Two researchers searched the reference lists of all selected studies for additional relevant articles. Disagreements between the two researchers were resolved by discussion. If a disagreement persisted, the judgment of a third researcher was considered decisive.

	Domain	Keywords
1	Transforming growth factor beta	(Transforming Growth Factor beta 1) OR (TGF-beta1) OR (Transforming Growth Factor-beta1) OR (TGF-beta-1) OR (TGF beta 1) OR (TGF-beta-2) OR (TGF beta 2) OR (TGFB2) OR (TGF-beta2) OR (TGF beta2) OR (TGF-beta-3) OR (TGF beta 3) OR (TGFB3) OR (TGF-beta3) OR (TGF beta3)
2	Oral submucous fibrosis	(oral submucous fibrosis*) OR (OSF) OR (collagen metabolism disorder*) OR (oral precancerous condition*)
3	Location	(buccal mucosa) OR (mouth mucosa) OR (oral mucosa) OR (cheek)
4	Immunohistochemistry	(Immunocytochemistry) OR (immunohistochemistry) OR (immunohistochemical staining) OR (immunolabeling technique*) OR (biochemical marker*) OR (biological marker*) OR (biomarker*)
5	Carcinoma	(malignant epithelial neoplasm*) OR (well differentiated squamous cell carcinoma) OR (SCC) OR (Carcinomas, Squamous Cell) OR (Squamous Cell Carcinomas) OR (Squamous Cell Carcinoma) OR (Carcinoma, Squamous)
6	Tranformation	(cell neoplastic transformation*) OR (tumorigenic transformation*) OR (malignant transformation*)

* Indicates wild card in PubMed

Figure 1: Search strategy

Data analysis

Two researchers utilised a standardised form to extract the relevant data. Any disagreements were resolved by discussion among the authors. For each selected study, the following data were then extracted: Author and year of publication, sample size, patient characteristics, country and study setting, study design, clinical staging, histopathological grading, technique, antibody, localisation, scoring criteria, results, epithelial expression and connective tissue expression of TGFβ, expression of TGF-β in normal mucosa, limitations of the included study, outcomes and inferences.

Role of funding source

There was no funding source for this study.

RESULTS

A total of 394 articles were retrieved for this review, including 334 from the databases and 60 from the additional sources. After eliminating duplicates, titles and abstracts of 264 articles were screened. Thirteen articles were eligible for full-text screening, of which three articles [Supplementary Table 2] did not meet the inclusion criteria. Ten full-text studies were finally identified, which included a study population of 579 patients histopathologically diagnosed with OSF and a control group of 131 healthy, non-diseased individuals for data extraction [Figure 2]. Tables 1 and 2 present the demographic data, OSF sampling, techniques used to evaluate TGF-β expression, findings, inferences and limitations of all included studies.

Description of studies

Ten studies met the inclusion criteria, of which seven studies were from India,[16-18,20,21,24,26] and one study each was from Bangladesh,[13] Sri Lanka[14]and China.[23] These studies were conducted in an institutional setting, and the study design was observational,[13,14,16-18,20,21,23,26] except for

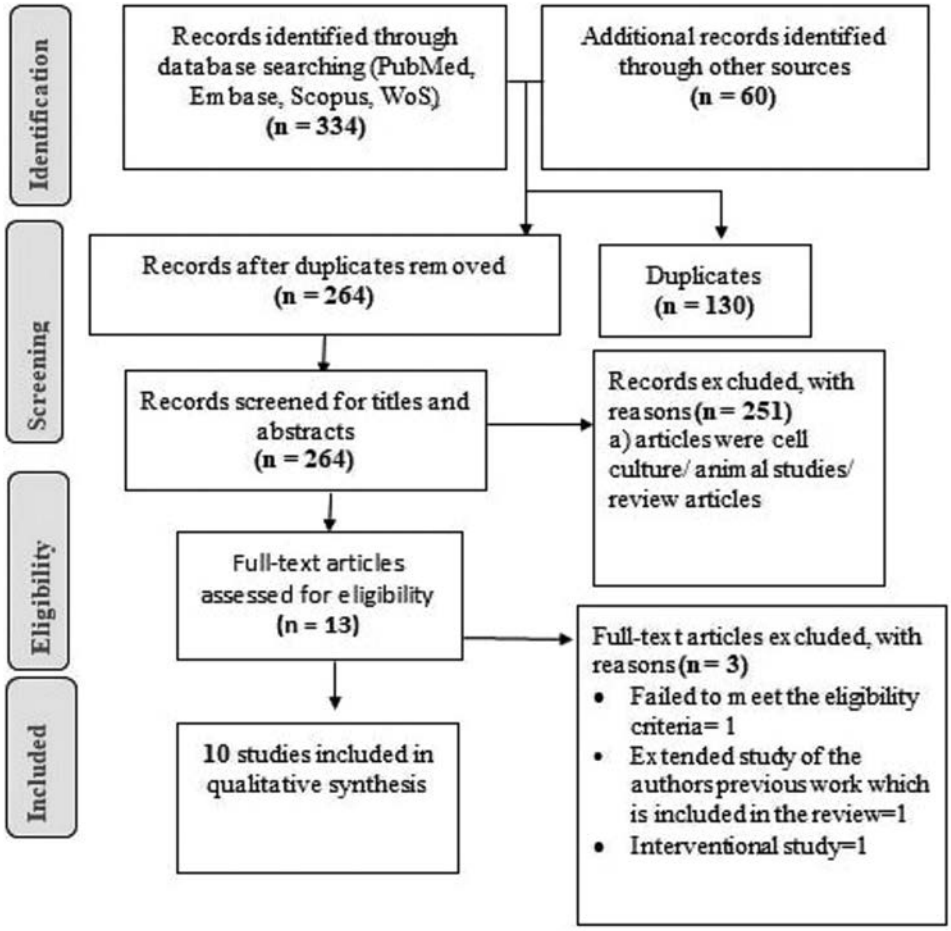

Figure 2: Flowchart summarising the article selection process (n-number of studies)

one cross-sectional study.[24] In the pooled study group of all included studies, the age of patients with OSF ranged from 16 to 72 years, with a higher population of males than that of females. Epidemiological data were not available in four studies.[17,18,20,23] Seven studies compared OSF cases with a control group,[13,14,16,17,20,23,26] and none of the studies included cases of oral squamous cell carcinoma (OSCC), occurring in a background of OSF, for the analysis.

With respect to OSF sampling, seven studies have used clinical staging/histological grading/both.[14,18,20,21,23,24,26] Out of these, three studies have discussed the results according to the initial grading system,[14,23,24] while three studies modified the classification for evaluation.[18,20,21] One study failed to discuss the stage-wise results.[26]

Studies on TGF-β

Three studies have assessed immunohistochemical expression of pan TGF-β and have reported 100% (30/30), 66.7% (56/84) and 75% (36/48) positivity in both epithelium and connective tissue of OSF samples.[13,18,21] A non-significant increase in pan TGF-β expression was observed in the early stages of OSF as compared to advance stages by Kale *et al*.[18] and Kumar *et al*.[21]

Studies on TGF-β1

Three studies have demonstrated positive and significantly upregulated TGF-β1 expression using polymerase chain reaction (PCR) in 50, 16 and 30 OSF samples each.[16,17,26] An (ELISA) enzyme-linked immunosorbent assay-based study on 73 OSF patients recorded highest TGF-β1 expression in the intermediate grades of OSF.[24] Five studies have reported 100% TGF-β1 immunopositivity in epithelium and connective tissue of 38, 11, 58, 71 and 30 OSF samples each.[14,17,20,23,26] An increase in TGF-β1 expression in the advanced stages of OSF was noted in three studies.[14,20,23]

Studies on TGF-β2

An upregulated TGF-β2 expression was demonstrated in two studies using PCR and IHC, in 70 and 30 OSF samples, respectively. One study found an increase in the advanced stages of OSF.[20]

Table 1: Demographic details, study setting, and OSF sampling details of the included studies

Author/ year of publication	Sample size		Age	Sex	Country and study setting	Study design	Clinical staging	Histopathological grading
	Study group	Control group						
Haque *et al*., 1998[13]	30	10	OSF=median 48 years (16–68) ; NOM=median 35 (19–45)	OSF=M-11, F-19; NOM=M-4, F-6	Bangladesh, institutional	Observational	ND	ND
Illeperuma *et al*., 2010[14]	38	8	OSF=21–65 years	OSF=M-36, F-6	Sri Lanka, institutional	Observational	ND	Utsunomiya *et al*. classification[15]
Rajendran *et al*., 2010[16]	50	50	OSF=mean 43.9 years, range 23–72	OSF=M: F ratio 2.6:1	India, institutional	Observational	ND	ND
Khan *et al*., 2011[17]	11	10	OSF=22–61 years; NOM= 10–35 years	OSF=M-4, F-7; NOM=F-5, M-5	India, institutional	Observational	ND	ND
	16	11	NS	NS	India, institutional	Observational	ND	ND
Kale *et al*., 2013[18]	84	ND	NS	NS	India, institutional	Observational	ND	Pindborg and Sirsat classification[19]
Kamath *et al*., 2015[20]	58	10	NS	NS	India, institutional	Observational	ND	Pindborg and Sirsat classification[19]
	70	10				Observational	ND	Pindborg and Sirsat classification[19]
Kumar *et al*., 2016[21]	48	ND	21–70 years	M: F=11:1	India, institutional	Observational	Khanna and Andrade classification[22]	Pindborg and Sirsat classification[19]
Wang *et al*., 2018[23]	71	12	NS	NS	China, institutional	Observational	Staging–primary, intermediate, or advanced	ND
Singh *et al*., 2019[24]	73	ND	47 were in the age group <30 years	M-88, F-12	India, institutional	Observational, cross-sectional. study conducted between January 2017 and September 2018	Based on mouth opening, cheek flexibility and tongue protrusion	Utsunomiya *et al*. classification[15]
Rai *et al*., 2020[26]	30	10	Mean age 31.96 years	M-27, F-3	India, institutional	Observational	Kerr *et al*. classification[27]	ND

OSF: Oral submucous fibrosis, TGF-β: Transforming growth factor beta, NOM: Normal, M: Male, F: Female, ND: Not done, NS: Not specified

Studies on TGF-β3

A single study reported statistically significant upregulation of TGF-β3 expression in OSF using PCR.[25,26]

Although the results were statistically analysed in all included studies, only three studies reported and discussed the findings based on the categorisation of OSF as per stages/grades that were originally allotted during sample selection.[14,23,24] The different classification systems, different methods of recording biomarker expression, as well as the large heterogeneity in terms of reporting of data made it difficult to conduct a proper systematic review of the extracted data. As the cutoff points used in the individual studies to determine positivity were most often the ideal cutoff values for each individual data set, pooling of data was not feasible as it may introduce bias and give an overestimation/underestimation of the actual degree of expression of TGF-β. For the aforementioned reasons, we could not perform a statistical analysis of the pooled data of all included studies in the present systematic analysis.

Risk of bias

The "a Cochrane risk of bias assessment tool: For non-randomised studies of interventions" (ACROBAT-NRSI) was used to assess the methodological quality of the included studies.[25] Two researchers separately assessed studies for significant, moderate, or low risk of bias (RoB) and a third researcher was conferred with in the event of disagreement.

The overall RoB for all the studies was low to moderate [Table 3]. Data related to confounding factors such as age, sex, and mouth opening were assessed, and a low RoB was observed in all the included studies.[13,14,16-18,20,21,23,24,26] The selection bias was assessed by evaluation of how the sample population was categorised and included in the study (based on clinical and/or histopathological classification system), and we found that seven studies had a low risk of bias,[14,18,20,21,23,24,26] and rest of the studies showed moderate bias.[13,16,17]

The measurement of intervention based on clonicity, origin and dilution of antibody were provided in seven studies.[13,14,16,20,23,24,26] Since, there were no changes in the technique during the study period and all the studies revealed the absence of discrepancy between the sample taken and the results discussed, the concerned RoB domain

Table 2: Technique specifications, limitations, outcome and inference of all included studies

Author/ year of publication	Technique	Antibody	Localisation	IHC scoring criteria	Results	Epithelial expression	Connective tissue expression	Normal mucosa	Limitations	Outcome	Inference
Haque *et al*., 1998[13]	IHC	TGF-β (1:300, rabbit)	NS	NS	30/30 (100%) positive	Positive	Positive in endothelial cells, T lymphocytes, monocytes, fibroblasts and platelets	Positive in connective tissue	Classification of OSF sample Clonicity, localisation and scoring criteria Epithelial cell distribution	Raised	Strong positive staining of the epithelium in OSF, whereas the normal epithelium was negative
Illeperuma *et al*, 2010[14]	IHC	TGF-β1 (1:50, rabbit, polyclonal)	NS	Frequency of positively stained cells assessed (<30%, 30%–70%, >70%)	38/38 (100%) positive	Positive in all layers except keratinised layer	Positive in submucosa; fibroblasts	Positive	Localisation	Increased in advanced stages	Significantly higher expression in OSF as compared to NOM
Rajendran *et al*., 2010[16]	PCR (blood)	TGF-β1 (7 polymorphisms)	NA	NA	Positive	NA	NA	Positive	Classification of OSF sample	The polymorphism in 5′UTR C-T in TGF-β1 gene has a significant association with OSF	Only one polymorphism significantly associated with OSF
Khan *et al*., 2011[17]	IHC	TGF-β1 (1:500)	Intercellular expression	Intensity of staining assessed based on a four-point scale of 0–3	11/11 (100%) positive	Positive in 9/11 cases	Positive in 10/11 cases	Positive	Classification of OSF sample Origin, clonicity and cell/tissue distribution Features of scoring criteria	Raised	Strong immunoreactivity in OSF tissues compared with mild-to-moderate staining in normal tissues
	PCR	TGF-β1	NA	NA	11/11 (100%) positive	NS	NS	Positive	Classification of OSF sample Cell/tissue utilised for PCR	Raised	Expression of TGF-β1 gene was found to be significantly increased in OSF compared with normal tissues
Kale *et al*., 2013[18]	IHC	TGF-β (anti-TGF-β1, β2, β3,) (1:200)	NS	Intensity of staining assessed (negative, mild, moderate, intense)	56/84 (66.7%) positive (early-22/24, advanced-34/60)	Positive in superficial and basal layer	Positive in fibroblast, macrophages, inflammatory cells, endothelial cells and deeper stroma	ND	Clonicity, origin and localisation Initial classification not followed for ease of evaluation	Early cases showed more intense TGF-β staining than the advanced cases	No statistically significant difference between early and advanced OSF cases
Kamath *et al*., 2015[20]	IHC	TGF-β1 (dilution- 4 μl/100 μl, mouse monoclonal)	NS	Intensity of staining and area of positivity assessed	58/58 (100%) positive	Positive in basal, suprabasal and spinous layer	Positive in submucosa, fibroblasts, inflammatory cells and endothelial cells, and muscle	Positive	Localisation Initial classification not followed for IHC scoring and analysis Grades I, II and III used for IHC analysis not defined	Increased with advanced stages	Significantly increased in OSF compared to NOM

Contd...

Table 2: Contd...

Author/ year of publication	Technique	Antibody	Localisation	IHC scoring criteria	Results	Epithelial expression	Connective tissue expression	Normal mucosa	Limitations	Outcome	Inference
Kumar *et al.*, 2016[21]	IHC	TGF-β	Cytoplasm	Staining intensity and staining area evaluated based on defined scoring criteria	36/48 (75%) positive	Positive in keratin, basal and spinous cells and basement membrane	Positive in fibroblast, endothelial cells and mast cells	ND	Clonicity, origin and dilution of marker Two classification combined to grade OSF samples	The mean TGF-β expression was greater and was of higher intensity in grades I/II than grade III	Non-significant difference among grades I, II and III.
Wang *et al.*, 2018[23]	IHC	TGF-β1 (1:200, rabbit)	Cytoplasm	Percentage of different positive cells using the formula (3+) × 3+(2+) × 2+(1+) × 1	71/71 (100%) positive	Positive	Positive in chronic inflammatory cells	Positive	Clonicity OSF sample staging criteria Epithelial distribution	Significantly lower expression in early/intermediate than advanced OSF	Significant difference between OSF and NOM
Singh *et al.*, 2019[24]	ELISA	TGF-β1	NA	NA	73/73 (100%) positive	NA	NA	ND	Missing reference for cheek flexibility and tongue protrusion criteria	Serum TGF-β1 levels were highest in the intermediate grades of OSF	Non-significant difference in both functional clinical stages and histopathological groups
Rai *et al.*, 2020[26]	IHC	TGF-β1 (1:1000, rabbit)	NS	Integrated density of staining	33/33 (100%) positive	Positive in basal and superficial layers	Positive in superficial and deeper layers	Negative	Clonicity and localisation Individual grade results	Raised	Significant difference between OSF and NOM
	PCR	TGF-β1, β2 and β3	NA	NA	Positive	NA	NA	Positive	Individual grade results	Raised	Statistically significant upregulation of TGF-β isoforms in OSF

OSF: Oral submucous fibrosis, TGF-β: Transforming growth factor beta, NA: Not applicable, ND: Not done, NS: Not specified, NOM: Normal mucosa, IHC: Immunohistochemistry, PCR: Polymerase chain reaction, ELISA: Enzyme-linked immunosorbent assay

Table 3: Risk of bias for all the included studies

Domain	Haque *et al*., 1998[13]	Illeperuma *et al*., 2010[14]	Rajendran *et al*., 2010[16]	Khan *et al*., 2011[17]	Kale *et al*., 2013[18]	Kamath *et al*., 2015[20]	Kumar *et al*., 2016[21]	Wang *et al*., 2018[23]	Singh *et al*., 2019[24]	Rai *et al*., 2020[26]
Bias due to confounding	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Bias in selection of participants into the study	Moderate risk	Low risk	Moderate risk	Moderate risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Bias in measurement of interventions	Low risk	Low risk	Low risk	Moderate risk	Moderate risk	Low risk	Moderate risk	Low risk	Low risk	Low risk
Bias due to departures from intended interventions	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Bias due to missing data	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Bias in measurement of outcomes	Moderate risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Bias in selection of the reported result	Moderate risk	Low risk	Moderate risk	Moderate risk	Low risk	Low risk	Low risk	Low risk	Low risk	Moderate risk
Overall	Moderate risk	Low risk	Moderate risk	Moderate risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk

was considered low risk.[13,14,16-18,20,21,23,24,26] The outcome measurement was low risk in nine studies,[14,16-18,20,21,23,24,26] and moderate risk in one study,[13] based on the localisation of markers, scoring criteria details and discussion of TGF-β expression in epithelial and connective tissue components. The bias in selecting the reported result was assessed based on the discussion of stage/grade-wise results and was found to be low in six studies,[14,18,20,21,23,24] and moderate in four studies.[13,16,17,26]

DISCUSSION

The present review identified an upregulated expression of TGF-β and isoforms in different stages of OSF. OSF has been established as a progressive disease, the identification of isoform-based targeted therapy in specific stages can modulate the pathogenesis of the disease. The transient presence of TGF-β is beneficial for tissue repair, whereas its persistent expression can lead to excessive fibrosis, crucial in OSF development [Figure 3].[1,28] The presence of TGF-β in the epithelium directs its role toward epithelial-mesenchymal transition (EMT). During the process, the transformed epithelial cells attain non-cohesiveness, evade polarity, and gain mesenchymal characteristics, thereby, exhibiting the ability to move and invade distant sites.[29]

TGF-β in connective tissue modulates the fibroblast phenotype and function in connective tissue, inducing myofibroblast transdifferentiation and promoting matrix accumulation. Their presence in endothelial cells induces endothelial-mesenchymal transition (EndoMT), which may later contribute to the development of fibrosis.[30] Studies on mature bovines have demonstrated that TGF-β induces and guides the transition of endothelial cells into myofibroblastic or smooth muscle phenotypes.[31] Additionally, fibrosis is the end result of the chronic inflammatory response, and TGF-β exhibits its dual nature as pro-inflammatory or anti-inflammatory cytokine under various circumstances. As OSF progresses from very early to advance stage, changes in the density of inflammatory cells can alter the expression of TGF-β.[32] Thus, researchers must specify the tissue component involved to decipher the mechanism of TGF-β involvement in OSF.

All of the included studies were conducted in South Asian countries,[13,14,16-18,20,21,23,24,26] where due to various cultural and geographical influences, consumption of areca nut is found to be higher.[33,34] The pooled age of the patients in the present study ranged from 16 to 72 years, with a male predilection.[13,14,16-18,20,21,23,24,26] The increase in the incidence of OSF in the younger population may be attributable to the easy availability and access to gutkha and pan masala, which contains areca nut.[35] This could also be the main factor resulting in the ethnic bias noted in the included studies.

Various techniques have been employed in human tissue, cell culture and animal studies to analyse TGF-β expression in OSF. The present systematic review includes molecular studies on the expression of TGF-β and its isoforms in human tissues *(in vitro)* in diagnosed cases of OSF.[13,14,16-18,20,21,23,24,26] IHC was the most commonly employed technique followed by PCR and ELISA. TGF-β1,[14,17,20,23,26] is the most commonly studied marker, followed by pan TGF-β,[13,18,21] and TGF-β2[20] with significantly high expression in all OSF cases compared to normal human buccal mucosa.[13,14,16,17,20,23,26]

In OSF, positive immunoexpression of pan TGF-β, TGF-β1 and TGF-β2 were observed in all epithelial layers with higher expression in basal layer followed by spinous layer[14,18,20,21,26] and in the connective tissue, fibroblasts, inflammatory cells, monocytes, platelets, collagen fibres, endothelial cells and muscles were

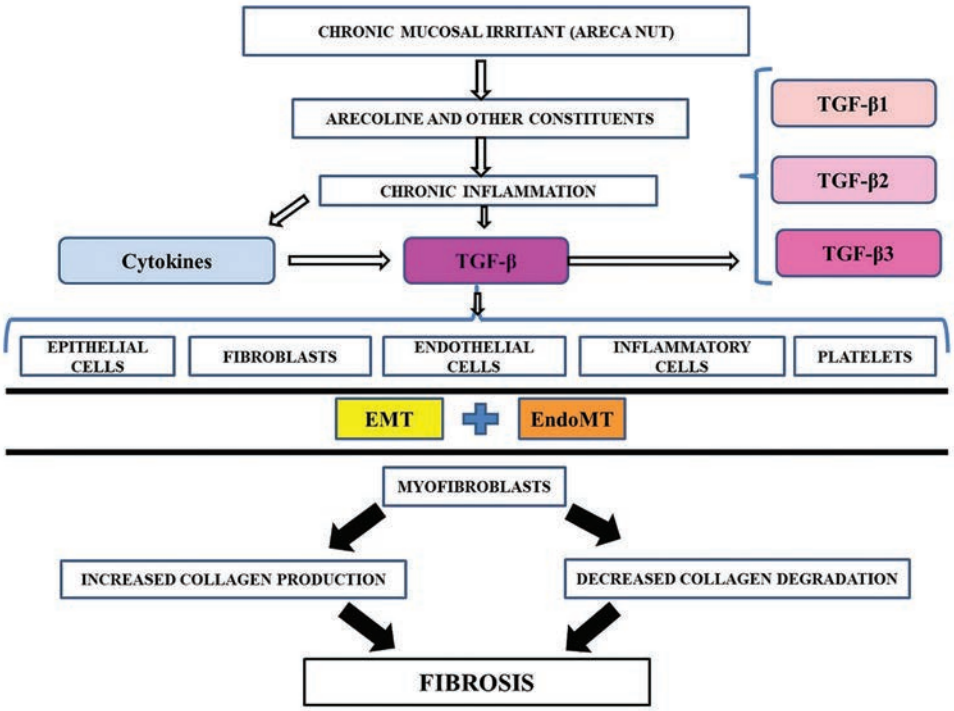

Figure 3: Role of TGF-β and its isoforms in the pathogenesis of OSF

positive.[13,14,18,20,21,23,26] Few researchers have failed to provide the specific cells and/or tissue involved in the epithelium,[13,17,23] and/or connective tissue.[17] Our review highlights the deficiency in reporting origin, clonicity, dilution and localisation of IHC marker. For a higher affinity and specificity to the epitope, rabbit antibodies show better reaction to immunogens compared to mice antibodies.[36] With respect to clonicity, monoclonal antibodies are more specific and target a single epitope, while polyclonal antibodies are more sensitive as they bind to multiple epitopes.[37] The dilution of the antibody defines the staining intensity, which is tested using an appropriate positive control,[37] and the results based on staining intensity can change with the change in dilution of the antibody. The processing errors and loss of antigenicity are few limitations of IHC, which directly affect the results and need to be addressed by researchers. Additionally, lack of a well-described scoring system questions the reproducibility of the results.

To identify differentially regulated genes in OSF, whole genome expression profiling strategy has been attempted. The PCR based studies have observed an upregulation of TGF-β and its isoforms in all the OSF tissue samples.[16,17,26]

Rajendran *et al*.[16] studied seven polymorphisms in the TGF-β1 gene and demonstrated that 5cUTRC-T polymorphism was significantly associated with OSF in comparison to the controls. These studies explained the genetic profiling of the disease and not the specific tissue involved (epithelium/connective tissue).

Approximately, 2%–8% of OSF cases have been reported to progress to OSCC.[28] Illeperuma *et al.* in their study, found no correlation between epithelial dysplasia in OSF and TGF-β expression.[14] However, the expression of TGF-β and its isoform has yet not been evaluated in OSF associated with malignant transformation.

To understand changes in TGF-β expression during the progression of the lesion, it is necessary to categorise OSF samples accurately. Seven studies grouped OSF samples using clinical staging and/or histopathological grading,[14,18,20,21,23,24,26] and six studies statistically analysed and discussed the difference in TGF-β expression based on different stages/grades of OSF.[14,18,20,21,23,24] An increased pan TGF-β expression was observed in the early stages of OSF,[18,21] while increased expression of TGF-β1 and TGF-β2 were noted in advanced stages of OSF.[14,20,23] However, these differences were not statistically significant. In OSF, early stages are predominantly inflammatory, whereas later stages are fibrotic. A reduced inflammatory component in later stages has been observed.[15,38]

The grading/staging of OSF is a crucial phenomenon that is often missed by researchers, especially when describing the results of the study groups leading to incomplete evaluation and discussion of TGF-β expression in OSF.

There are various OSF classifications, the relative merits of which are confusing.[38] No direct correlation between histopathological grading and clinical staging was noted, which adds to the dilemma of the OSF sampling procedure. The degree and extent of fibrosis vary depending on the oral mucosa and muscle region, indicating the need for universally accepted OSF classification which minimises disagreement, reduces sampling error and provides a common platform for discussion.

Regarding the therapeutic use of TGF-β, Shi *et al*.[39] found that specific blockading of TGF-β1 reduces chances of random suppression of pan TGF-β signalling pathways. It also has fewer adverse effects on the tumour microenvironment and is safer than pan TGF-β blocking. Therefore, clarity around isoform expression pattern could provide guidance for the design of selective targeting of TGF-β drugs.

This systematic review had certain limitations. First, the number of included studies, as well as the included study population (patients with OSF) are relatively small. We included only human studies on OSF, which led to the exclusion of several studies that, although reported important aspects, did not fulfill the eligibility criteria. However, we considered several different databases and languages and our search provided an extensive number of hits (over 330). Moreover, we also manually searched the references of included articles. Secondly, seven of ten studies are based on Indian population, and all ten studies are based on Asian population. Therefore, we could not account for geographical or racial differences in the review, and the findings are not generalisable and should therefore be treated with caution. Based on the world health organisation statistics, there are more than five million OSF patients globally.[40] Resultantly, whether the expression of TGF-β and its isoforms in Western patients are identical with Asian ones is still unknown. Thirdly, the differences in the techniques and reporting styles of the included articles that met the inclusion criteria as aforementioned, considerably limited the process of synthesis of the findings. However, we have attempted to present a fair summarisation of the relevant findings of all included articles to elucidate the pattern of TGF-β expression in OSF.

CONCLUSION

The articles included in the review had moderate quality, homogenous data, and similar methodology. These studies have shown a statistically significant increase in the expression of TGF-β in OSF when compared with normal healthy mucosa. Additionally, an increased pan TGF-β expression was noted in the early stages of OSF whereas elevated TGF-β1 and TGF-β2 expressions were seen in the later stages of OSF. However, more information on TGF-β3 in OSF is needed. The results of our systematic review should be confirmed with additional relevant research in the future using updated analyses.

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Conflicts of interest
There are no conflicts of interest.

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SUPPLEMENTARY TABLES

Supplementary Table 1A: Search strategy for Embase

Domains	Keywords
Transforming growth factor beta	'Transforming growth factor beta' OR 'transforming growth factor beta 1' OR 'transforming growth factor beta 3' OR 'tgf beta 1' OR 'tgf beta 3' OR 'tgfb1' OR 'tgfb3' OR 'transforming growth factor beta superfamily proteins' OR 'platelet derived transforming growth factor beta'
Oral submucous fibrosis	'Oral submucous fibrosis' OR 'OSF' OR 'collagen metabolism disorder' OR 'oral precancerous condition' OR 'oral potentially malignant disorder' OR 'mouth disease'
Location	'Buccal mucosa' OR 'mouth mucosa' OR 'oral mucosa' OR 'cheek'
Immunohistochemistry	'Immunocytochemistry' OR 'immunohistochemistry' OR 'immunohistochemical staining' OR 'immunolabeling technique' OR 'biochemical marker' OR 'biological marker' OR 'biomarker' OR 'antigen staining'
Carcinoma	'Malignant epithelial neoplasm' OR 'well differentiated squamous cell carcinoma' OR 'SCC' OR 'carcinomas, squamous cell' OR 'squamous cell carcinomas' OR 'squamous cell carcinoma' OR 'carcinoma, squamous'
Transformation	'Carcinomatous degeneration' OR 'degeneration, malignant' OR 'malignant degeneration' OR 'malignant transformation' OR 'cell neoplastic transformation' OR 'tumorigenic transformation'

Supplementary Table 1B: Search strategy for Scopus

Domains	Keywords	Notes
Transforming growth factor beta	"Transforming growth factor beta I" OR "TGF-beta1" OR "transforming growth factor-beta1" OR "TGF-beta-1" OR "TGF beta 1" OR "TGF-beta-3" OR "TGF beta 3" OR "TGFB3" OR "TGF-beta3" OR "TGF beta3"	…
Oral submucous fibrosis	"Oral submucous fibrosis*" OR "OSF" OR "collagen metabolism disorder*" OR "oral precancerous condition*"	Fibrosis, fibroses, disorders, conditions
Location	"buccal mucosa" OR "mouth mucosa" OR "oral mucosa" OR "cheek"	-
Immunohistochemistry	"Immunocytochemistry" OR "immunohistochemistry" OR "immunohistochemical staining" OR "immunolabeling technique*" OR "biochemical marker*" OR "biological marker*" OR "biomarker*"	Techniques, technic(s), markers
Carcinoma	"Malignant epithelial neoplasm*" OR "well differentiated squamous cell carcinoma" OR "SCC" OR "carcinomas, squamous cell" OR "squamous cell carcinomas" OR "squamous cell carcinoma" OR "carcinoma, squamous"	Neoplasms
Transformation	"Cell neoplastic transformation*" OR "tumorigenic transformation*" OR "malignant transformation*"	Transformations

Supplementary Table 1C: Search strategy for all databases

Databases	Search strategy	Filters used	Total searches
PubMed	#1 AND #2 AND #3 AND #6	ALL FIELDS	4
PubMed	#1 AND #2 AND #3 AND #4	ALL FIELDS	12
PubMed	#1 AND #2 AND #3 AND #4AND #5 AND #6	ALL FIELDS	3
PubMed	#1 AND #3 AND #4	ALL FIELDS	92
PubMed	#1 AND #2 AND #4 AND #5	ALL FIELDS	5
Embase	#1 AND #2 AND #3 AND #6	ALL FIELDS	2
Embase	#1 AND #2 AND #3 AND #4	ALL FIELDS	10
Embase	#1 AND #3 AND #4	ALL FIELDS	67
Embase	#1 AND #2 AND #4 AND #5	ALL FIELDS	23
Scopus	#1 AND #2 AND #3 AND #6	Title-Abstract-Keyword	5
Scopus	#1 AND #2 AND #3 AND #4	Title-Abstract-Keyword	30
Scopus	#1 AND #3 AND #4	Title-Abstract-Keyword	58
Scopus	#1 AND #2 AND #4 AND #5	Title-Abstract-Keyword	23
Searches from Google Scholar and other sources			60
Total			394

SUPPLEMENTARY TABLES

PRISMA 2020 Checklist [10]

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Title
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Abstract
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Introduction
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Introduction
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Search strategy and selection paragraph
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Search strategy and selection paragraph
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Search strategy and selection paragraph
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened eachrecord and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Search strategy and selection paragraph
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they workedindependently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used inthe process.	Search strategy and selection paragraph
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain ineach study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	NA
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describeany assumptions made about any missing or unclear information.	NA
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessedeach study and whether they worked independently, and if applicable, details of automation tools used in the process.	Risk of bias paragraph
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	NA
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristicsand comparing against the planned groups for each synthesis (item #5)).	Data extraction
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or dataconversions.	NA
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	NA

	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe themodel(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	NA
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	NA
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	NA
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Risk of bias paragraph

PRISMA 2020 Checklist [10]

Section and Topic	Item #	Checklist item	Location where item is reported
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	NA
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studiesincluded in the review, ideally using a flow diagram.	Flowchart, search strategy
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Flowchart, search strategy Supplementary table
Study characteristics	17	Cite each included study and present its characteristics.	Table 1 and 2
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Table 3
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and itsprecision (e.g. confidence/credible interval), ideally using structured tables or plots.	NA
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	NA
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision(e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	NA
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	NA
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	NA
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	RoB paragraph
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	NA
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Discussion section
	23b	Discuss any limitations of the evidence included in the review.	Discussion section
	23c	Discuss any limitations of the review processes used.	Discussion section
	23d	Discuss implications of the results for practice, policy, and future research.	Discussion section
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	PROSPERO [CRD42022319970]

	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Protocol can be accessed
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Declaration of funding
Competing interests	26	Declare any competing interests of review authors.	Declaration of competing interests
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted fromincluded studies; data used for all analyses; analytic code; any other materials used in the review.	NA