Myofibroblasts in oral potentially malignant disorders: is it related to malignant transformation?

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Running title: Myofibroblasts in oral potentially malignant disorders.

Abstract

In oral cancer, acquisition of α-smooth muscle actin (α-SMA)-positive fibroblasts, known as myofibroblasts or carcinoma-associated fibroblasts (CAF), is an important event for progression and metastasis. However, the contribution of myofibroblasts in oral potentially malignant disorders (OPMD) remains controversial. This systematic review provides evidence that immunodetection of myofibroblasts may identify oral submucous fibrosis (OSMF) with high-risk of malignant transformation, but does not represent an auxiliary tool to predict the malignant potential of leukoplakia and erythroplakia, the most common OPMD.

Introduction

Oral cancer, most represented by oral squamous cell carcinoma (OSCC), is one of the 10 most prevalent cancers worldwide and poses a significant health threat, with only 50% of patients surviving more than 5 years. The 2012 world estimation showed an annual incidence of 300,000 new cases (2.1% of the total cancers) and approximately 145,000 deaths due to the disease (Ferlay *et al*, 2015). A series of oral disorders are well-recognized to precede OSCC, including leukoplakia, erythroplakia, oral submucous fibrosis (OSMF) and actinic cheilitis (AC), whereas the potential of others, such as oral lichen planus (OLP), is still somewhat questionable. However, in a recent meta-analysis, Aghbari and colleagues (2017) pooled 19676 OLP cases from 57 studies and showed an overall transformation to oral cancer in 1.1% of cases, which was higher among smokers, alcoholics and HCV-infected patients. The identification and treatment of those lesions, called oral potentially malignant disorders (OPMD), is essential to minimize or even eliminate the risk of malignant transformation. However, not all disorders are amenable to curative treatment, and the malignant transformation does not occur in every single lesion. Therefore, the identification of biomarkers for transformation is of great importance.

As stated by Professor Scully, the presence and the severity of epithelial dysplasia are considered the main parameters related to malignant transformation of the OPMD, but the histological assessment of the epithelial dysplasia is a source of substantial subjectivity (Scully, 2014). There are also numerous molecular markers reported to be associated with the malignant transformation of OPMD, but to date there is no single marker that reliably can be used to predict the transformation of those disorders.

Recent data show that exchange of cytokines, extracellular matrix proteins and enzymes between tumor and stromal cells induce the development and progression of tumors. An important feature of OSCC is the acquisition of α-smooth muscle actin (α-SMA)-positive fibroblasts, termed myofibroblasts or carcinoma-associated fibroblasts

(CAF), which, in turn, induce the proliferation and invasion of the tumor cells (Sobral *et al*, 2011; Kabir *et al*, 2016). The contribution of myofibroblasts in OPMD remains controversial, with some reporters describing myofibroblasts in the stroma of those lesions and correlating the density of them with the severity of epithelial dysplasia (Chaudhary *et al*, 2012; Joshi *et al*, 2016), but others did not confirm it (Kellermann *et al*, 2007; Etemad-Moghadam *et al*, 2009). In OSMF, myofibroblasts are more consistently found, mainly in dysplastic lesions, and in vitro studies have revealed that myofibroblasts may play an important role in pathogenesis of disease (Chang *et al*, 2014; Pant *et al*, 2016). In order to clarify the value of myofibroblasts in transformation of OPMD, this article carried out a systematic review of literature including publications addressing the importance of immunodetection of myofibroblasts for OPMD transformation.

Methods

Immunohistochemical studies assessing the prognostic relevance of myofibroblasts (α-SMA-positive fibroblasts) in OPMD samples were systematically reviewed at Cochrane, Lilacs, PubMed, Scopus and Web of Science databases. The search strategy was developed combining the following terms: (1) oral potentially malignant disorder or oral potentially malignant lesion or leukoplakia or erythroplakia or oral submucous fibrosis or actinic cheilitis or oral lichen planus or oral dysplasia and (2) myofibroblast or carcinoma-associated fibroblast or α-SMA-positive fibroblast or α-SMA-positive cell. The search was conducted on March 28th, 2017. All hits were retrieved and examined, discarding duplicates. After careful browsing of the title and abstract, unrelated studies were excluded. References of the selected studies were also searched for relevant studies. The following information was collected: author, year of publication, country, number and characteristics of samples and results.

Results

A total of 134 studies were initially identified, but after exclusion of duplicates and of irrelevant studies to the analysis, 19 articles were included in this systematic review (Table 1). Oral dysplasias clinically represented by AC and leukoplakia/erythroplakia were included in 1 and 13 studies, respectively. OSMF was present in 7 studies, and 1 study included OLP. Thirteen studies included normal oral mucosa for comparison, whereas 2 studies included fibrous hyperplasia with normal epithelium and 1 study included hyperkeratosis. Three studies detected myofibroblasts in the stroma of the normal oral mucosa (Angadi *et al*, 2011; Sarode *et al*, 2016; Yu *et al*, 2016). Out of 189 normal oral mucosa samples, 20 (10.6%) samples were positive for myofibroblasts, 16 showing scarce cells and 4 with an abundant number of cells. Hyperkeratosis was included in 1 study (Seifi *et al*, 2010) and out of 18 samples, 1 revealed few myofibroblasts in the subjacent stroma. One study that included fibrous hyperplasia with normal epithelium as a control group showed lack of myofibroblasts in the subjacent stroma (Rodrigues *et al*, 2015), whereas the other revealed sparse number of positive cells (Vered *et al*, 2009).

Out of 263 oral dysplasias, myofibroblasts were found in 19 (7.2%) samples, mainly in a scanty and focal pattern. Only two studies (Chaudhary *et al*, 2012; Joshi *et al*, 2016), accounting for 13 samples, found a significant increase of myofibroblasts in high grade/severe dysplasia in comparison with normal oral mucosa. A proportion meta-analysis revealed no significant difference ($p=0.21$) in presence of myofibroblasts between normal oral mucosa (20/169, positive/negative samples) and oral dysplasias (19/244). The only study conducted with AC (Bianco *et al*, 2015) showed positivity for myofibroblasts in all samples, regardless the intensity of dysplasia. For OLP, no myofibroblasts were found in the stroma of a cohort containing 32 samples (Parajuli *et al*, 2007).

In OSMF, of 213 cases from 6 studies, 188 (88.3%) were positive for myofibroblasts. Four studies (Angadi *et al*, 2011; Gupta *et al*, 2015; Sarode *et al*, 2016;

Yu *et al*, 2016) demonstrated that the presence of myofibroblasts is significantly higher in OSMF compared with normal oral mucosa. A statistical significance was also noted between advanced OSMF (cases with dysplasia) compared to early OSMF (cases without dysplasia), as reported by Angadi *et al* (2011), Rao *et al* (2014) and Sarode *et al* (2016). The pooled analysis showed that density of myofibroblasts was significantly higher in advanced OSMF (10 classified as negative/low expression versus 50 classified as moderate/high) compared with early OSMF (73 samples classified as negative/low expression versus 18 classified as moderate/high) ($p<0.0001$).

Discussion

The malignant transformation of OPMD is a clinically important step in oral tumorigenesis, yet poorly understood. The importance of changes in the microenvironment during tumor progression has been increasingly recognized, but in OPMD, the participation of myofibroblasts seems lesion-dependent. In leukoplakias and erythroplakias (the most common OPMD in the largest part of the world), which are traditionally associated with smoking tobacco, epithelial dysplasia was not associated with the presence of myofibroblasts. Indeed, only a small proportion of cases was classified as positive for myofibroblasts (7.2%), and no association between severity of dysplasia and myofibroblasts was observed. In the studies that identified myofibroblasts in the stroma of those OPMD (Vered *et al*, 2009; Seifi *et al*, 2010; Chaudhary *et al*, 2012; Gupta *et al*, 2015; Joshi *et al*, 2016), the immunohistochemical images provided by the authors are not sufficiently clear to distinguish smooth muscle cells from blood vessels of myofibroblasts or stromal reaction due to superficially invasive OSCC. Therefore, acquisition of myofibroblasts seems not related to transformation of leukoplakias and erythroplakias into OSCC. Furthermore, the underlying connective tissue of oral dysplasias is characterized by different levels of inflammatory response, which tend to be inversely correlated with acquisition of myofibroblasts (Kellermann *et al*, 2007; Dayan *et al*, 2012). This is corroborated by the

findings that myofibroblasts are rarely found in early-stage OSCC, indicating that the direct contact between tumor cells and resident fibroblasts is necessary for transformation in myofibroblasts (Kawashiri *et al*, 2009; Kelner *et al*, 2015).

AC, an OPMD of the lips, is caused by exposure to ultraviolet radiation and demonstrates a transformation rate of approximately 20% (Markopoulos *et al*, 2004). It is difficult to treat because surgical treatments may result in significant esthetical and functional side effects and conservative procedures show uncertain efficacy. The only study evaluating the potential participation of myofibroblasts in the progression of AC revealed that myofibroblasts were presented in all cases regardless the transformation potential (Bianco *et al*, 2015). As detailed by the authors, this finding may be explained by the basophilic degeneration of the connective tissue present in most of the lesions due to excessive exposure to ultraviolet radiation leading to degeneration of extracellular matrix proteins (elastosis) and deregulation of the resident fibroblasts of the underlying connective tissue. However, future studies are essential to elucidate the participation of myofibroblasts on AC transformation into lip squamous cell carcinoma.

The malignant transformation of OLP to OSCC has always been a subject of great controversy, however, the recent meta-analysis of Aghbari and colleagues (2017) found an overall transformation in 1.1% of cases. Consistently, the inclusion only of the studies that applied the strict WHO diagnostic criteria of 2003, accounting to 3803 cases, revealed a transformation potential of 0.9%. Unfortunately, there is only one study evaluating myofibroblasts in OLP (Parajuli *et al*, 2017) and, of 32 cases, none revealed α-SMA-positive myofibroblasts. This finding is not unexpected, since OLP is a common chronic inflammatory disease characterized by a disequilibrium in the expression patterns of various inflammation-related cytokines, including transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ) (Lu *et al*, 2015) - master keys on myofibroblast activation (Sobral *et al*, 2007). While TGF-β induces fibroblast-myofibroblast transformation, IFN-γ blocks this process via stimulation of SMAD7 and

inhibition of connective tissue growth factor (CTGF), which has been considered the effective promoter of myofibroblast activation (Sobral *et al*, 2011).

OSMF is mainly restricted to Southeast Asia, but some cases have been also described in other parts of the world. Although the exact pathogenesis of OSMF remains unclear, the understanding of the cellular and molecular mechanisms involved in producing the tissue alterations are evolving. On this context, some in vitro and in vivo studies have demonstrated the participation of myofibroblasts on OSMF. All studies addressing the immunodetection of α-SMA-positive cells in OSMF revealed the presence of myofibroblasts. Myofibroblasts were detected in more than 88% of the studied samples, revealing that an increase in myofibroblasts was positively associated with OSMF at advanced stage, those with increased tendency to malignant transformation. Tumor cells induce myofibroblast activation through synthesis of some cytokines and growth factors such as TGF-β (Kellermann *et al*, 2008). Interestingly, in vitro studies demonstrated that areca nut constituents induce TGF-β release by normal epithelial cells and concomitantly promote the transformation of normal oral fibroblasts into myofibroblasts (Chang *et al*, 2014; Pant *et al*, 2016), providing evidence of the putative mechanism by areca nut leads to OSMF. Together, acquisition of myofibroblasts in the stroma of OSMF is suggestive of malignant transformation of OSMF. However, the mechanisms leading to acquisition of myofibroblasts are far from being understood.

In closing, the results of this review article point to an important role of myofibroblasts in OSMF, which may serve as a reliable marker to identify lesions with high-risk of malignant transformation. However, the lack of myofibroblasts in most of the leukoplakia and erythroplakia indicates that immunodetection of myofibroblasts may not represent an auxiliary tool for prediction of malignant potential of those OPMD. With only one study assessing myofibroblasts in AC and OLP, the evidence is inconclusive. It is worth noting that all studies included in this review article examined OPMD without a continuous follow up from their diagnosis to malignant transformation,

thus the question remains whether they would have or not transformed into OSCC. This fact certainly warrants further analysis.

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Author contribution

Both authors have conceived, written and revised the manuscript.

Conflicts of interest

None to declare.

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Table 1. Overview of the presence of myofibroblasts (α-SMA-positive fibroblasts) in oral potentially malignant disorders.

Study	Year	Oral samples	Presence	Comment
Kellermann *et al* (Brazil)	2007	18 normal mucosa 16 leukoplakia with dysplasia	No	
Etemad-Moghadam *et al* (Iran)	2009	15 normal mucosa 15 dysplasia	No	
Vered *et al* (Israel)	2009	16 fibrous hyperplasia 12 mild dysplasia 11 moderate to severe dysplasia	Yes	Sparse to absent in both fibrous hyperplasia and dysplasia
Seifi *et al* (Iran)	2010	18 hyperkeratosis 18 dysplasia	Yes	Scanty in 1 sample of hyperkeratosis and focal in 4 cases of oral dysplasia
Angadi *et al* (India)	2011	15 normal mucosa 35 early OSMF 35 advanced OSMF	Yes	Positivity in 2 normal mucosa and in OSMF, but higher in advanced cases of OSMF
Chaudhary *et al* (India)	2012	15 normal mucosa 15 low-risk dysplasia 15 high-risk dysplasia	Yes	Only in high-risk dysplasia (46.66% of the cases)

De-Assis *et al* (Brazil)	2012	10 normal mucosa	No	
		30 leukoplakia with dysplasia		
Fujii *et al* (Japan)	2012	5 normal epithelia	No	
		24 dysplasia		
Kapse *et al* (India)	2013	21 severe dysplasia	No	
Rao *et al* (India)	2014	41 OSMF without dysplasia	Yes	Higher frequency in OSMF with dysplasia
		10 OSMF with dysplasia		
Rodrigues *et al* (Brazil)	2015	29 fibrous hyperplasia	No	
		24 mild dysplasia		
		26 moderate dysplasia		
		19 severe dysplasia		
Jayaraj *et al* (India)	2015	32 normal mucosa	Yes	Positivity in 2 cases of OSMF
		5 mild dysplasia		
		5 moderate dysplasia		
		6 severe dysplasia		
		3 early OSMF		
		13 advanced OSMF		

Gupta *et al* (India)	2015	10 normal mucosa 14 dysplasia 11 OSMF	Yes	Positivity in dysplasia and OSMF, with higher expression in OSMF
Bianco *et al* (Brazil)	2015	9 actinic cheilitis with mild dysplasia 14 actinic cheilitis with moderate dysplasia 7 actinic cheilitis severe dysplasia	Yes	Found in all samples regardless intensity of dysplasia
Yu *et al* (Taiwan)	2016	15 normal mucosa 35 OSMF	Yes	Positivity in all cases of both normal mucosa and OSMF
Sarode *et al* (India)	2016	10 normal mucosa 15 early OSMF 15 advanced OSMF	Yes	Significantly higher density in advanced OSMF compared with normal mucosa and early OSMF
Joshi *et al* (India)	2016	10 normal mucosa 7 mild dysplasia 8 moderate dysplasia 5 severe dysplasia	Yes	Normal mucosa and mild dysplasia were negative, whereas 1 sample of moderate dysplasia and 5 of severe dysplasia were classified as scanty
Anura *et al* (India)	2017	10 normal mucosa 10 OSMF	Yes	Positivity only in OSMF

Parajuli *et al* (Norway)	2017	24 normal mucosa 5 dysplasia 32 oral lichen planus	No

OSMF: oral submucous fibrosis.