Original Article

Role of programmed cell death 4 in myofibroblast differentiation in oral submucous fibrosis

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Abstract

Background: Fibrosis is an uncontrolled healing process, led by persistent differentiation of fibroblast to alpha-smooth muscle actin (αSMA) positive activated fibroblast or myofibroblast. Oral submucous fibrosis (OSMF) is one such condition that is associated with areca nut use. Recently, Programmed Cell Death 4 (PDCD4), a pro-apoptotic marker, has been shown to modulate fibroblast differentiation in various organ fibrosis. The present study aimed to evaluate the role of PDCD4 in the regulation of fibroblast differentiation in OSMF.

Materials and Methods: Paraffin-embedded tissue sections from 45 cases of the normal oral mucosa, early OSMF and advanced OSMF were examined for PDCD4 and αSMA expression by immunostaining. Co-expression of PDCD4 and αSMA in fibroblasts was examined using Spearman's correlation test.

Results: The stromal fibroblasts showed minimal expression of αSMA in the normal mucosa and early OSMF, while advanced OSMF groups demonstrated a higher frequency of αSMA myofibroblasts. The PDCD4 expression was noted in the normal stromal fibroblasts. However, this expression appeared to progressively reduce with an increasing grade of OSMF. Thus, a negative correlation was noted between stromal PDCD4 and αSMA expression with progressive OSMF.

Conclusion: This study demonstrated a putative role for PDCD4 in oral fibrosis consistent with its role in other tissues. The lack of PDCD4 expression with increasing myofibroblast expression in OSMF suggests that targeting its dysregulation may be an attractive translational therapeutic target.

Keywords: Areca, betel nut, myofibroblast, oral submucous fibrosis, programmed cell death

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INTRODUCTION

Oral submucous fibrosis (OSMF) constitutes an oral premalignant condition induced by areca nut ingestion and is considered an uncontrolled tissue healing response resulting in fibrosis.[1] Clinically, OSMF presents with a burning sensation in the mouth, stiffening of the mucosa, and reduced mouth opening, among other changes that lead to difficulty in food consumption and generalized

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debilitation. Histologically, it is associated with progressive changes in the connective tissue such as fibrosis and hyalinization, along with atrophy of the epithelium in advanced cases. The connective tissue response to arecoline, a major alkaloid of areca nut, is known to up-regulate pro-inflammatory and pro-fibrotic cytokines, such as Transforming Growth Factor-beta (TGF-β). TGF-β leads to the differentiation of stromal fibroblasts to alpha smooth muscle actin positive (αSMA) myofibroblasts that are well known to promote matrix synthesis and tissue contraction.[2,3] Recently, alkaloids of arecanut, namely arecaidine and guvacine, along with the polyphenols such as catechin and tannins, have also been shown to induce TGF-β signaling in epithelial cells contributing to the complex pathogenesis of OSMF.[4,5]

Programmed cell death 4 (PDCD4) is a well-known pro-apoptotic protein recognized for its roles in transcription and translation pathways in tumor growth and metastasis. PDCD4 has been observed to promote fibroblasts differentiation in renal and liver fibrosis through TGF-β and other regulatory pathways.[6-8] In a recent study, we observed reduced PDCD-4 expression in oral dysplastic and oral carcinomas compared to normal epithelial mucosa.[9] The decrease in PDCD4 expression in transforming epithelia appeared to correlate well with reduced cell death observed in these lesions. A key reason for the persistence of myofibroblast phenotypes has been their ability to evade apoptosis.[10] Thus, the premise for this study was to examine if the changes in PDCD4 expression would correlate with increased differentiation of oral stromal fibroblasts into αSMA positive myofibroblasts. We investigated αSMA and PDCD4 expression in the normal and progressive stage of OSMF oral mucosa to assess its putative role in tissue fibrosis.

MATERIALS AND METHODS

Human tissue samples

Following ethical clearance from the Institute, archival formalin-fixed, paraffin-embedded tissues of normal mucosa (n = 10), and OSMF (n = 30) were retrieved. Clinical data were used to evaluate the history with areca nut consumption and for the absence of other local and systemic factors or illnesses.

Histological analyses

Three continuous sections of 4 um were cut, and one section was stained using hematoxylin and eosin. Histological grading from I through IV was based on criteria elaborated by Pindborg and Sirsat.[1] Cases were then categorized into two groups as either early OSMF for histological Grades I and II cases (Group 2) or advanced OSMF for histological Grade III and IV cases (Group 3) and compared to normal mucosa (Group 1).

Immunostaining

Two sections were used for immunohistochemistry with PDCD4 (Clone EPR3432, BioGenex Lab) and αSMA (clone EP188, PathnSitu). The primary antibody was detected using a one-step polymer HRP kit (PEH2, PathnSitu) by the Avidin-Biotin complex and counterstained with Harris's hematoxylin.[11]

Immunostaining analyses

Sections were analyzed for the staining intensity, localization and pattern by two independent examiners. The two examiners had similar levels of experience with training in immunohistochemical and pathological evaluation of oral lesions. The inter-observer agreement using a training set was 0.89 (Kappa statistic). For study groups, any discrepancy in scoring was resolved by the consensus on a multi-head microscope.

A modified scoring criterion was adopted due to the compression and amassing of the cells noted in histological sections in OSMF. To compare the expression of PDCD4 and aSMA in fibroblasts, consecutive sections were stained and analyzed. Subjective errors on identifying cell types such as endothelial cells, inflammatory cells and fibroblast were reduced by confirmed at high magnification prior to scoring. For αSMA expression, the intensity of positive cells was scored as 0 for no staining, 1 for cells visible at high power and 2 for cells visible at medium power. For the αSMA expression pattern, scoring criteria by Etemad-Moghadam *et al.* were modified as follows: 0-absent to 1, 2 or 3 for low expression and 4, 5 or 6 for high expression of αSMA^{+} cells.[12] The intensity for PDCD4 expression was scored as 0 for no staining, 1 for mild to moderate brown, 2 for dark brown as per modified Reis and Tomenson criteria.[13] To assess percentage of expression for both αSMA and PDCD4, percentage of positively stained fibroblast were scored as 0 = 0%–10% positive cells, 1 = 11%–30% positive cells, 2 = 31%–60% positive cells and 3 = 60%–100% positive cells per field. A cumulative total score representing the intensity and percentage was expressed as 0, 1 and 2 indicated low/normal expression pattern while 3, 4 and 5 indicated overexpression pattern.

Statistical analyses

Expression data for PDCD4 and αSMA were tabulated in Excel and analyzed in SPSS (version 16.0, IBM, Seattle, Washington, USA) for statistical significance. To test the association between the two, the Chi-square and Fisher

exact tests were performed, and their correlation was determined using the Spearman's correlation test.

RESULTS

αSMA expression and localization within connective tissue cells

All the cases of normal mucosa and 10 of 15 (66.7%) of early OSMF cases demonstrated a low frequency of fibroblasts expressing αSMA [Figure 1a]. Amongst these, eight cases of normal mucosa presented with an absence of staining for αSMA fibroblasts. Total expression of αSMA showed similar results in normal mucosa and early OSMF (P = 0.061) [Table 1]. In the advanced OSMF group, 13 out of 15 cases (86.7%) presented with a high frequency of αSMA myofibroblasts. The frequency of αSMA myofibroblast between normal mucosa and early OSMF versus advanced OSMF groups was statistically significant (P = 0.001 and P = 0.0008, respectively) [Table 1]. On examining their localization, αSMA^+ myofibroblasts were present within superficial connective tissue and epithelial-connective tissue junction in early OSMF cases [Figure 1b]. In advanced OSMF cases, the localization varied and could be appreciated even in the deeper stroma [Figure 1c]. Taken together, normal mucosa and early OSMF cases presented with a low frequency of αSMA^+ fibroblasts, while advanced OSMF exhibited high frequency (P = 0.001) [Table 2]. These results clearly established the preponderance of αSMA^+ myofibroblasts with progressive OSMF stages.

Programmed cell death 4 expression and localization within connective tissue cells

Next, we examined PDCD4 intensity and pattern in these tissues. PDCD4 expression is predominantly limited to the nucleus, with low cytoplasmic expression in normal cells. We observed positive staining for fibroblasts in all three groups [Figure 1d-f]. Nuclear PDCD4 staining in the epithelium in normal and early OSMF was evident, while it appears to be predominantly cytoplasmic in advanced OSMF. A significant reduction in total PDCD4 expression in early OSMF (P = 0.05) and advanced OSMF (P = 0.001) was observed compared to normal mucosa [Table 3]. PDCD4 expression among the OSMF groups appeared to be similar. Next, we examined the pattern of PDCD4 expression in these tissues and noted lower percentage of positive cells with increasing grade of fibrosis. Overall, the

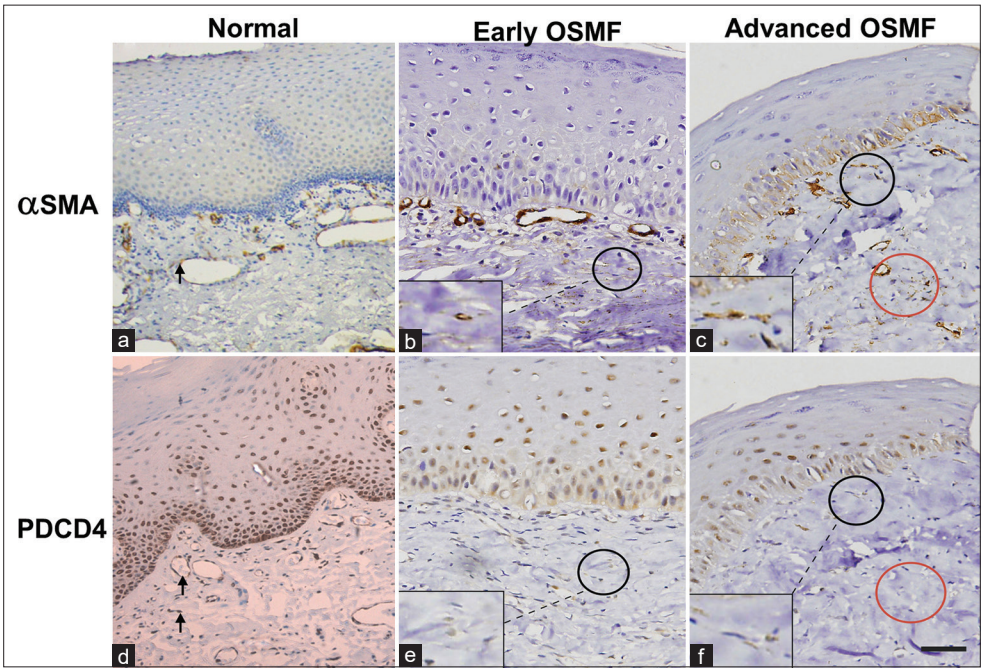


Figure 1: Consecutive histological sections of normal oral mucosa, early and advanced Oral submucous fibrosis stained with αSMA, and programmed cell death 4. (a) Photomicrograph of normal oral mucosa stained with aSMA with positive cytoplasmic staining of αSMA (arrows), predominantly in a perivascular location; (b) Photomicrograph of early Oral submucous fibrosis showing aSMA positive fibroblasts in the connective tissue stroma with a high-power magnification (black circle and inset image); (c) Photomicrograph of advanced Oral submucous fibrosis showing aSMA positive fibroblasts in the juxtaepithelial (black circle) and deeper connective tissue stroma (red circle), inset shows high-power image with prominent αSMA expression; (d) Photomicrograph of normal oral mucosa stained with Programmed Cell Death 4 showing epithelium, stromal fibroblasts (marked by arrow) and a nuclear and peri-vascular localization; (e) Photomicrograph of early oral submucous fibrosis showing mild programmed cell death 4 expression in stromal fibroblasts (black circle and inset image); (f) Photomicrograph of advanced oral submucous fibrosis showing few programmed cell death 4 positive fibroblasts throughout superficial (black circle) and deep connective tissue (red circle), inset shows high-power image noting lack of prominent staining

differences in PDCD4 staining among the three groups were strikingly significant ($P = 0.001$) [Table 4]. These observations established a reduced expression of PDCD4 were consistent with prior observations noting PDCD4 in tissue fibrosis.

Correlating αSMA and programmed cell death 4 expression in fibroblasts

Finally, to examine the αSMA and PDCD4 expression in fibroblasts, we compared immunostaining in the consequent section in each case. Normal oral mucosa and advanced OSMF demonstrated an inverse relationship between αSMA and PDCD4 staining using Spearman's rank correlation (correlation coefficient = −0.71) [Table 5].

DISCUSSION

Oral tissues are well known to be minimally fibrotic, leading to comparisons with fetal-like nonscarring wounds.[14-16] The significant immune-active surveillance, including the role of saliva in the oral cavity, has been correlated with a mitigated inflammatory response that appears to shift the tissue healing response to a nonscarring resolution. Several studies have examined the role of matrix molecules (small leucine proteoglycans), adhesion molecules (integrins αVβ6, Connexin 43, and CD 44), and growth factor isoforms (TGF-β3 versus β1) in mediating this phenotype.[17-22] It appears to be a critical teleological adaption that enables normal oral functions due to injury and rapid healing necessary for normal oral physiological functions. In this context, an oral disease with prominent clinical manifestation of fibrosis, as evident in OSMF is a striking dichotomy from the normal oral pathophysiological responses. Normal oral healing has transient expression of myofibroblasts, analogous to cutaneous wounds but has been demonstrated to involve minimal matrix synthesis and contraction.[14] Myofibroblasts play key, permissive roles in

Table 1: Comparison of the alpha-smooth muscle actin expression of fibroblasts in normal oral mucosa, early oral submucous fibrosis, and advanced oral submucous fibrosis cases using the Fischer's exact test

Groups	αSMA expression (intensity + percentage)			*P*		
	Low	High	Total			
Normal mucosa (1)	10 (100)	0	10 (100)	1 versus 2	-	1 versus 3
Early OSMF (2)	10 (66.7)	5 (33.3)	15 (100)	0.061 (NS)	2 versus 3	0.008*
Advanced OSMF (3)	2 (13.3)	13 (86.7)	15 (100)	-	0.001*	

Fisher's exact test, *$P \leq 0.05$, PDCD4 expression scores: 0-2 low, 3-5 high. NS: Not significant, αSMA: Alpha-smooth actin, PDCD4: Programmed cell death 4, OSMF: Oral submucous fibrosis

Table 2: Comparison of the alpha-smooth muscle actin expression pattern of fibroblasts in normal oral mucosa, early oral submucous fibrosis, and advanced oral submucous fibrosis cases using Chi-square test

Groups	αSMA pattern (intensity + percentage)			*P*
	Low/normal expression	Over expression	Total	
Normal mucosa (1)	10 (100)	0	10 (100)	0.001*
Early OSMF (2)	10 (66.7)	5 (33.3)	15 (100)	
Advanced OSMF (3)	2 (13.3)	13 (86.7)	15 (100)	

Chi-square test, *$P \leq 0.05$, PDCD4 expression scores: 0-2 low, 3-5 high. αSMA: Alpha-smooth actin, PDCD4: Programmed cell death 4, OSMF: Oral submucous fibrosis

Table 3: Comparison of Programmed Cell Death 4 expression of fibroblasts in normal oral mucosa, early oral submucous fibrosis, and advanced oral submucous fibrosis cases using the Fischer's exact test

Groups	PDCD4 expression (intensity + percentage)			*P*		
	Low	High	Total			
Normal mucosa (1)	0	10 (100)	10 (100)	1 versus 2	-	1 versus 3
Early OSMF (2)	6 (40)	9 (60)	15 (100)	0.050*	2 versus 3	0.001*
Advanced OSMF (3)	11 (73.3)	4 (26.7)	15 (100)	-	0.139 (NS)	

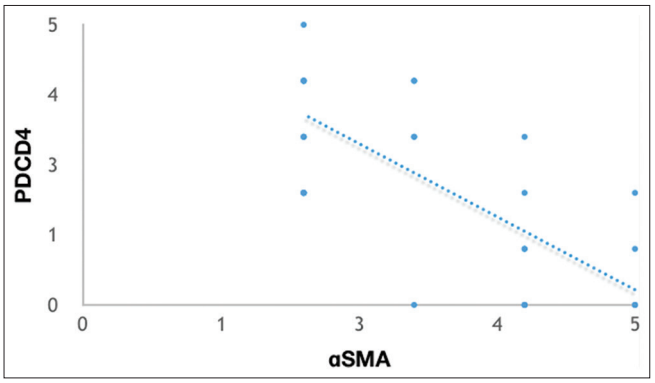
Fisher's exact test, *$P \leq$ *stt, PDCD4 expression scores: 0-2 low, 3-5 high. NS: Not significant, PDCD4: Programmed cell death 4, OSMF: Oral submucous fibrosis

Table 4: Comparison of Programmed Cell Death 4 expression pattern of fibroblasts in normal oral mucosa, early oral submucous fibrosis, and advanced oral submucous fibrosis cases using Chi-square test

Groups	PDCD4 pattern (intensity + percentage)			*P*
	Low/normal expression	Over expression	Total	
Normal mucosa (1)	0 (0)	10 (100)	10 (100)	0.001*
Early OSMF (2)	6 (40)	9 (60)	15 (100)	
Advanced OSMF (3)	11 (73.3)	4 (26.7)	15 (100)	

*$P \leq 0.05$, Chi-square test, PDCD4 expression scores: 0-2 low, 3-5 high. PDCD4: Programmed cell death 4, OSMF: Oral submucous fibrosis

Table 5: Correlation between Programmed Cell Death 4 and alpha-smooth muscle actin expression of fibroblasts in the study groups using Spearman Rank Correlation Test



Group	Correlation coefficient	Interpretation
Overall	−0.71	High negative correlation

PDCD4: Programmed cell death 4, αSMA: Alpha-smooth actin

the progression of malignant tissues.[23,24] Given the reported premalignant potential for OSMF, the role of myofibroblasts in determining its biological behavior has important clinical implications.

Recent advances in matrix biology have unraveled a broad heterogeneity in fibroblasts within connective tissue.[25] While the precise origin of the myofibroblasts remains unclear, phenotypic similarities have suggested that they arise from peri-vascular (pericyte) and stromal fibroblasts.[26] A key signaling pathway, TGF-β1, has been well established as a primary inducer of the αSMA differentiated myofibroblast phenotype.[5] Further, several downstream signaling intermediates such as HiC5, miR21, and PDCD4 have been implicated in the TGF-β driven process leading to organ fibrosis and tumor stroma modulation.[6-8,27-29] Among them, PDCD4 is known to regulate cell apoptosis and has roles in the activated fibroblast phenotype. The presence of nuclear (active) versus cytoplasmic localization of PDCD4 reflects its functional state in various cell types.[30] Loss of PDCD4 expression is associated with increased cell survival and changes in cellular functions. However, differences in apoptosis sensitivity and rate cell turnover of oral versus cutaneous cells have been well reported.[31]

This study was aimed at examining the role of PDCD4 and myofibroblasts in OSMF. As anticipated, there was a distinct increase in the number of αSMA myofibroblasts in progressive OSMF stages, as reported previously.[32-34] Moreover, we noted PDCD4 expression in normal mucosa and early OSMF was predominantly nuclear in the stromal fibroblasts. Interestingly, the advanced cases of OSMF showed either low or absence of nuclear PDCD4 expression in the stromal fibroblasts. Further, consecutive sections stained with PDCD4 and αSMA demonstrated a high negative correlation in advanced OSMF cases. These results are consistent with recent reports by Zang *et al.* and Sun *et al.,* who reported αSMA myofibroblasts lacked PDCD4 expression in hepatic and renal fibrosis, respectively.[7,8] They implicate the lack of PDCD4 expression to a TGF-β driven miR-21 and activation protein-1 feedback loop during myofibroblast transformation. Thus, our findings are in congruence and support the hypothesis that PDCD4 plays a central role in myofibroblast differentiation leading to tissue fibrosis.

OSMF represents both features of a fibrotic stroma and an atrophic epithelial component with discrete histological and clinical courses in stark contrast to oral squamous cell carcinomas.[35,36] Recent reports have emphasized that the OSCC arising in preexisting OSMF has a more verrucous and ulcero-hyperproliferative nature with an indolent clinical course.[36-38] These together suggest that while OSMF is a degenerative (epithelial atrophy) and aberrant healing (stromal fibrosis) response, a secondary crucial transformative event is necessary for malignancy. The chronic, sustained exposures of epithelial components to the injurious agent (areca nut, tobacco, or combinations) along with permissive changes in the underlying stroma such as fibrosis and hypoxia may contribute to the low but consistent, premalignant potential of OSMF. While the key molecular event mediating this transformation remains to be fully investigated, a potential candidate could be PDCD4 with its low, cytoplasmic expression evident in the OMSF epithelium as well as other dysplastic, premalignant oral lesions.[9,13] These observations are supported by the well-documented tumor suppressor role of PDCD-4 in carcinomas from multiple anatomical sites such as cervical, colorectal, endometrial, breast, pancreatic, prostate, gastric, brain, esophagus, lung and ovarian cancers, among others.[39-47] Thus, PDCD4 expression in oral premalignant lesions, including OSMF in their epithelial compartment, could potentially useful for prognostication of clinical risk, protracted surveillance, and monitoring.

CONCLUSION

This study noted a negative correlation of PDCD4 expression with increased myofibroblast differentiation in OSMF. Future investigations can focus on therapeutically targeting the TGF-β regulated miR21-PDCD4-mediated stromal signaling for OSMF management.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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